



Comparative effects of the parasiticide ivermectin on survival and reproduction of adult sepsid flies[☆]

Sheena Conforti¹, Jana Dietrich¹, Thierry Kuhn¹, Nicola van Koppenhagen¹, Julian Baur, Patrick T. Rohner, Wolf U. Blanckenhorn^{*}, Martin A. Schäfer

Department of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

ARTICLE INFO

Keywords:

Drug resistance
Dung insects
Ecotoxicology
Exaptation
Ivermectin
Maternal effects
Mortality
Oviposition choice
Paternal effects
Phylogenetic signal
Reproduction
Sepsidae

ABSTRACT

Ivermectin is a veterinary pharmaceutical widely applied against parasites of livestock. Being effective against pests, it is also known to have lethal and sublethal effects on non-target organisms. While considerable research demonstrates the impact of ivermectin residues in livestock dung on the development and survival of dung feeding insect larvae, surprisingly little is known about its fitness effects on adults. We tested the impact of ivermectin on the survival of adult sepsid dung fly species (Diptera: Sepsidae) in the laboratory, using an ecologically relevant and realistic range of 69–1978 µg ivermectin/kg wet dung, and compared the sensitivities of larvae and adults in a phylogenetic framework. For one representative, relatively insensitive species, *Sepsis punctum*, we further investigated effects of ivermectin on female fecundity and male fertility. Moreover, we tested whether females can differentiate between ivermectin-spiked and non-contaminated dung in the wild. Adult sepsid flies exposed to ivermectin suffered increased mortality, whereby closely related species varied strongly in their sensitivity. Adult susceptibility to the drug correlated with larval susceptibility, showing a phylogenetic signal and demonstrating systemic variation in ivermectin sensitivity. Exposure of *S. punctum* females to even low concentrations of ivermectin lowered the number of eggs laid, while treatment of males reduced egg-to-adult offspring survival, presumably via impairment of sperm quality or quantity. The fitness impact was amplified when both parents were exposed. Lastly, sepsid flies did not discriminate against ivermectin-spiked dung in the field. Treatment of livestock with avermectins may thus have even more far-reaching sublethal ecological consequences than currently assumed via effects on adult dung-feeding insects.

1. Introduction

Over the past decades, research has shown that the use of agrochemicals, in particular pesticides, can have lethal as well as sublethal effects on beneficial, non-target arthropods including pollinators, detritivores, and natural enemies (Desneux et al., 2007; Henry et al., 2012). Insecticides have been shown to impact on feeding and oviposition behaviour, physiology, development, longevity, fecundity and sex ratios (Desneux et al., 2007). These effects ultimately modify the abundance and species composition, impeding important ecosystem functions (Zhang et al., 2007; Lu et al., 2012; Floate et al., 2016). Although less widely recognized, a class of substances with potentially

severe and increasing ecotoxicological impact are human and veterinary pharmaceuticals, which can affect arthropod diversity in aquatic as well as terrestrial habitats (Fent et al., 2006; Schmitt and Römbke, 2008). Given the drastic and ongoing decline in insect diversity and abundance (Hallmann et al., 2017), understanding effects of pharmaceuticals and their residues on insects is of utmost necessity.

In this study we investigate the impact of ivermectin, a chemical compound commonly used in veterinary and human pharmaceuticals applied against parasitic nematodes and arthropods such as ticks and lice (Campbell et al., 1983; Ōmura, 2008). Treated mammals cannot metabolize ivermectin completely. Therefore, ivermectin residues are regularly present in livestock faeces where they have been shown to

[☆] All authors conceived and designed the study, and all contributed to the statistical analysis and the writing of the manuscript. SC, JD, TK & NvK largely performed the experiments. JB, MAS & PTR took care of the fly cultures for extended periods of time.

^{*} Corresponding author.

E-mail addresses: sheena.conforti@uzh.ch (S. Conforti), jana.dietrich@uzh.ch (J. Dietrich), thierry.kuhn@uzh.ch (T. Kuhn), nicola.vankoppenhagen@uzh.ch (N.v. Koppenhagen), julian.baur@ebc.uu.se (J. Baur), patrick.rohner@uzh.ch (P.T. Rohner), wolf.blanckenhorn@uzh.ch (W.U. Blanckenhorn), martin.schaefer@ieu.uzh.ch (M.A. Schäfer).

¹ Shared first authors, as all contributed equally.

Table 1

Origin of the species used, LC50 values, and statistical results from the Cox mixed-effects model with random error.

	Population origin	LC50 larvae [µg ivermectin/kg wet dung]	LC50 adult [µg ivermectin/kg wet dung]	Single-species models		
				Hazard ratio	SE [ln(HR)]	P
<i>Sepsis cynipsea</i>	Zurich, CH	0.36	644.62	1.002032	2.62e – 4	< 0.01
<i>S. duplicata</i>	Zurich, CH	0.09	2342.55	1.001502	2.57e – 4	< 0.01
<i>S. flavimana</i>	Zurich, CH	0.05	2915.50	1.001171	2.55e – 4	< 0.01
<i>S. fulgens</i>	Zurich, CH	5.68	9961.35	1.000399	4.21e – 4	0.340
<i>S. lateralis</i>	Tenerife, E	0.80	608.53	1.001865	2.81e – 4	< 0.01
<i>S. neocynipsea</i> NA	Montana, USA	0.23	1603.52	1.000758	2.11e – 4	< 0.01
<i>S. neocynipsea</i> EU	Sörenberg, CH	1.57	1203.71	1.001679	3.09e – 4	< 0.01
<i>S. orthocnemis</i>	Zurich, CH	1.09	4196.52	1.000583	2.11e – 4	< 0.01
<i>S. punctum</i> NA	Ottawa, CAN	1.66	1801.68	1.000572	2.81e – 4	< 0.05
<i>S. punctum</i> EU	Zurich, CH	4.24	2985.37	1.001136	2.70e – 4	< 0.01
<i>S. thoracica</i>	Zurich, CH	0.64	794.55	1.001645	3.29e – 4	< 0.01

have pronounced effects on many non-target, often beneficial organisms that dwell in and feed on dung, thereby breaking it down (Campbell et al., 1983; Herd et al., 1996; Floate, 1998; Gonzalez-Tokman et al., 2017a). Binding to γ -aminobutyric acid and glutamic acid receptors, ivermectin augments the membrane permeability for chloride ions, thus interfering with the organisms' nervous and muscular systems, particularly during moult (Schaeffer and Turner, 1989). Recent experiments investigating the performance of multiple non-target organisms showed that ivermectin sensitivity is a synapomorphy of all ecdysozoa (moulting invertebrates, comprising nematodes and arthropods) (Puniamoorthy et al., 2014).

Previous research has focused mainly on the effects of ivermectin on insect larvae (Lumaret et al., 2012). This is because larvae of many (but certainly not all) dung insects consume (contaminated) dung, and toxicologists and regulators are typically interested primarily, if not exclusively, in the direct mortality effects of toxic substances. Nevertheless, sublethal effects at lower ivermectin concentrations must and indeed do occur, for instance in terms of prolonged development, suboptimal growth, or stunted body size (Römbke et al., 2009). Such sublethal effects impede individual performance in various ways directly and indirectly, well known effects in community ecology that are relevant from a biological perspective (e.g. TerHorst et al., 2015). Moreover, many dung organisms rely on dung as food not only as larvae but also as adults (Skidmore, 1991), and different life-stages may vary in sensitivity. Consequently, the impact of environmental toxins on biodiversity and ecosystem functioning are likely systematically underestimated when ignoring sublethal effects and focussing exclusively on juvenile life-stages (Lumaret et al., 2012; Gonzalez-Tokman et al., 2017a).

We here used black scavenger (or dung) flies (Diptera: Sepsidae) to study the fitness consequences of ivermectin for adult dung feeding insects, thus extending previous studies that investigated its effects on their juvenile development and mortality (Madsen et al., 1990; Floate, 1998; Iwasa et al., 2005; Blanckenhorn et al., 2013). We were particularly interested in whether feeding on ivermectin-spiked dung influences adult longevity and offspring production at realistic, ecologically relevant concentrations that remain detectable in the field for long time periods after cattle treatment (Liebig et al., 2010). To address this issue, we exposed adult flies of eleven (sub)species to fresh dung spiked with varying ivermectin concentrations and determined their mortality. If ivermectin sensitivity is indeed an evolvable species-specific physiological property as suggested earlier (Puniamoorthy et al., 2014), we expected that juvenile and adult ivermectin sensitivity should covary across the sepsid phylogeny. In one representative but relatively insensitive species, *Sepsis punctum*, we further tested whether ivermectin affects female fecundity and/or male fertility even when offspring are subsequently raised in untreated dung. We also investigated whether males exposed to ivermectin change their mating behaviour, and whether flies can differentiate between contaminated and

uncontaminated dung under natural conditions, complementing our previous common garden laboratory research on larvae (Blanckenhorn et al., 2013).

2. Methods

2.1. Ivermectin treatments

In all experiments, we used dung originally collected from grass-fed cattle that had not been recently treated with parasiticides. The dung was subsequently homogenized and frozen at -80°C for several weeks. Six ivermectin concentrations were prepared following a semi-logarithmic scale and covering the wide range of concentrations reported as residues in nature because the substance is rather inert (Liebig et al., 2010): $C_{70} \approx 69$; $C_{300} \approx 269$; $C_{700} \approx 692$; $C_{800} \approx 833$; $C_{1000} \approx 1008$; $C_{2000} \approx 1978$ μg ivermectin/kg wet dung. The ivermectin solution was thoroughly mixed into wet dung and kept overnight at room temperature to allow for evaporation of the solvent acetone. A standard acetone treatment was used as control (C_0).

2.2. Comparative adult longevity

We worked with offspring of 11 (sub)species of black scavenger flies of the genus *Sepsis* that were originally caught in the wild on or around cow dung at various places (Table 1). Laboratory cultures were established in 1-l plastic containers using offspring of at least 10 wild-caught gravid females and thereafter kept in replicate groups in the laboratory for multiple generations prior to our experiment. Fly groups were regularly supplied with fresh cow dung, sugar, and water ad libitum using standard methods. Seven species were represented by one population, and we additionally studied two continental populations each of the widespread *Sepsis punctum* (Zurich (CH) and Ottawa (USA)) and *Sepsis neocynipsea* (Zurich (CH) and Montana (USA)). New and Old World populations of both species differ in life history, morphology and mating system and can thus be treated as independent evolutionary lineages (Puniamoorthy et al., 2012; Rohner et al., 2016, 2018).

We experimentally exposed adult flies of all 11 taxa to ivermectin-spiked fresh dung to assess effects on longevity. Immediately after emergence flies were haphazardly removed from laboratory cultures and continuously exposed to either an acetone control (C_0) or one of five different ivermectin concentrations (C_{300} , C_{700} , C_{800} , C_{1000} , C_{2000}). In each replicate, eight adult flies were kept in a 1-litre plastic container with dry sugar and a plastic dish ($20 \times 40 \times 15 \text{ mm}^3$) filled with ca. 10 g of spiked dung. Each treatment was replicated five times (i.e. $n = 5 \times 8 = 40$ flies per treatment and taxon). All test containers were placed in a climate chamber set at 24°C , 60% relative humidity and 14 h light, and over the four subsequent days mortality was assessed every 24 h.

For the statistical analysis of adult mortality we used Cox mixed-

effects regression using the package “coxme”, version 2.3 in R (R Development Core Team, 2008; Therneau and Grambsch, 2013). Models were run for each species separately, as well as for the combined dataset in which species identity was added as an additional factor, allowing to test for a treatment by species interactions.

We further estimated the concentration of ivermectin at which 50% of all adults died over the 4-day observation period ($LC50_{adult}$ [in μg ivermectin/kg fresh dung]) following standard procedures (Floate et al., 2016). These estimates were then used to test for an association across species between larval and adult susceptibility to ivermectin by regressing the $LC50$ values estimated for adults against those estimated previously for their larvae (Puniamoorthy et al., 2014). As larval mortality caused by ivermectin previously exhibited a pronounced phylogenetic signal, we accounted for the relatedness among species using a phylogenetic generalized linear model (PGLS; R-package “caper” version 0.5.2: Orme et al., 2013). We used the cladogram published by Zhao et al. (2013), setting all branch lengths to unity. Note that Puniamoorthy et al. (2014) at times provided $LC50$ values for multiple populations per species, in which case we chose the estimate of the population geographically closest to our study population.

2.3. Fecundity and fertility of *Sepsis punctum*

We next studied the effects of exposure to ivermectin-contaminated dung in adult European *S. punctum* by assessing the number of eggs laid by females (fecundity) and egg-to-adult survival of the offspring (which is additionally a function of male sperm transfer and function). Newly-emerged virgin flies were kept in single-sex containers with sugar and exposed to either an acetone control (C_0) or one of three ivermectin treatments (C_{70} , C_{300} , C_{700}). After 3 days of exposure to spiked dung, $n = 60$ pairs of each of the ten treatment combinations (Table 2) were randomly assembled and held at constant 18°C in glass vials containing a dish of untreated dung for oviposition ($20 \times 20 \times 15 \text{ mm}^3$) and sugar for food. Over a period of 5 days, eggs were counted daily under a binocular microscope, whereafter pairs were transferred to new vials equipped with fresh dung. Individual clutches were subsequently incubated at 24°C and kept at a 14 h light cycle. After emergence, offspring were counted to quantify egg-to-adult survival.

We analysed the probability of females laying eggs, the number of eggs laid, the probability of offspring emergence, and offspring egg-to-adult survival as a function of the maternal and paternal ivermectin treatments (Table 2). In all cases, we treated ivermectin concentrations as an (ordered) factor and tested for interactive effects between maternal and paternal treatment. If not significant ($P > 0.1$), this interaction term was removed. To analyse the probability of a female laying eggs, a binomial general linear mixed model (GLMM) was used that additionally controlled for maternal body size (hind tibia length) measured after death. The number of eggs laid was analysed using a GLMM again including maternal body size as covariate (because larger females generally lay more eggs). A binomial GLMM was used to model the probability of offspring to emerge with the number of eggs as covariate. Egg-to-adult survival of offspring was analysed using a GLMM with underlying binomial error structure. If necessary,

Table 2

Experimental setup of the fecundity experiment with various ivermectin treatment combinations of male and female flies ($n = 60$ pairs per treatment combination).

♀	♂			
	C_0	C_{70}	C_{300}	C_{700}
C_0	$C_0 \times C_0$	$C_0 \times C_{70}$	$C_0 \times C_{300}$	$C_0 \times C_{700}$
C_{70}	$C_{70} \times C_0$	$C_{70} \times C_{70}$		
C_{300}	$C_{300} \times C_0$		$C_{300} \times C_{300}$	
C_{700}	$C_{700} \times C_0$			$C_{700} \times C_{700}$

experimental blocks were added as random effect. All analyses were conducted in R, using the *lmer*-package (Bates et al., 2015).

2.4. Mating behaviour of *Sepsis punctum*

We assessed the influence of adult ivermectin exposure on male mating behaviour by measuring the mating latency and copulation duration of European *S. punctum*. Only male flies were exposed to ivermectin because copulation duration in sepsid flies is largely controlled by the male (Martin and Hosken, 2002). There were two treatments: an acetone control and one (intermediate) ivermectin concentration (C_{700}). As above, groups of 15 males were held in containers equipped with dishes ($20 \times 40 \times 15 \text{ mm}^3$) containing ca. 10 g of either spiked or uncontaminated dung as well as sugar at 18°C for three days. For the behavioural assay, control ($n = 28$) and ivermectin-exposed males ($n = 35$) were thereafter paired individually with a random untreated female in a glass vial containing a smear of dung to stimulate mating and observed for 60 min. Male copulation frequency was compared with binary logistic regression, and copulation duration and mating latency with Mann-Whitney *U*-tests.

2.5. Oviposition choice in the field

In our field oviposition experiment we used an acetone control (C_0) and four ivermectin concentrations (C_{70} , C_{300} , C_{700} , C_{2000}) at each replicate location. Dung spiked with the various ivermectin-acetone solutions was filled into round plastic petri dishes (10 (depth) \times 55 (diameter) mm^2). Five plastic dishes containing dung of all above treatments were randomly clustered together over an area of roughly $50 \times 50 \text{ cm}^2$ at $n = 10$ locations in the field surrounding our University (Irchelpark), where the opportunistic *S. punctum* is the most abundant sepsid species (but other species occur as well). After five hours, all eggs with the diagnostic, genus-specific morphology were counted. We analysed variation in the total number of eggs laid as a function of ivermectin treatment with a non-parametric Kruskal-Wallis test.

3. Results

3.1. Comparative longevity of ivermectin-feeding adult sepsid flies

Adult mortality risk increased with ivermectin concentration in all eleven taxa (Fig. 1; Table 1). This effect further differed between taxa (significant taxon \times ivermectin treatment interaction: $X^2 = 40.06$; $P < 0.001$).

On average, $LC50$ values were much higher (by at least three orders of magnitude) for adults than for larvae (mean and SE across species: $LC50_{adults} = 2641.63 \pm 807$ vs. $LC50_{larvae} = 1.49 \pm 0.55 \mu\text{g}$ ivermectin/kg wet dung). Species that were more strongly affected by ivermectin at the juvenile stage (cf. Puniamoorthy et al., 2014) were also more susceptible to ivermectin as adults ($r_{PGLS} = 0.76$ [0.30, 0.89] 95% CI, $P < 0.01$), although this pattern was strongly driven by the relatively resistant species of the *punctum* group (including *S. punctum*, *S. fulgens* and *S. orthocnemis*: Figs. 1, 2).

3.2. Fecundity and fertility of *Sepsis punctum* feeding on contaminated dung

The probability of females laying eggs decreased when they were exposed to higher ivermectin concentrations, whereas male ivermectin treatment showed no significant effect on egg laying probability (Fig. 3; Table 3A). Among the females that successfully laid eggs, the number of eggs also decreased with increasing ivermectin concentrations, an effect that was again unrelated to male treatment (Table 3B).

The probability of offspring emergence decreased with maternal as well as paternal exposure to ivermectin (interaction not significant and thus removed; Fig. 4; Table 3C). Furthermore, offspring egg-to-adult survival decreased with increasing maternal and paternal ivermectin

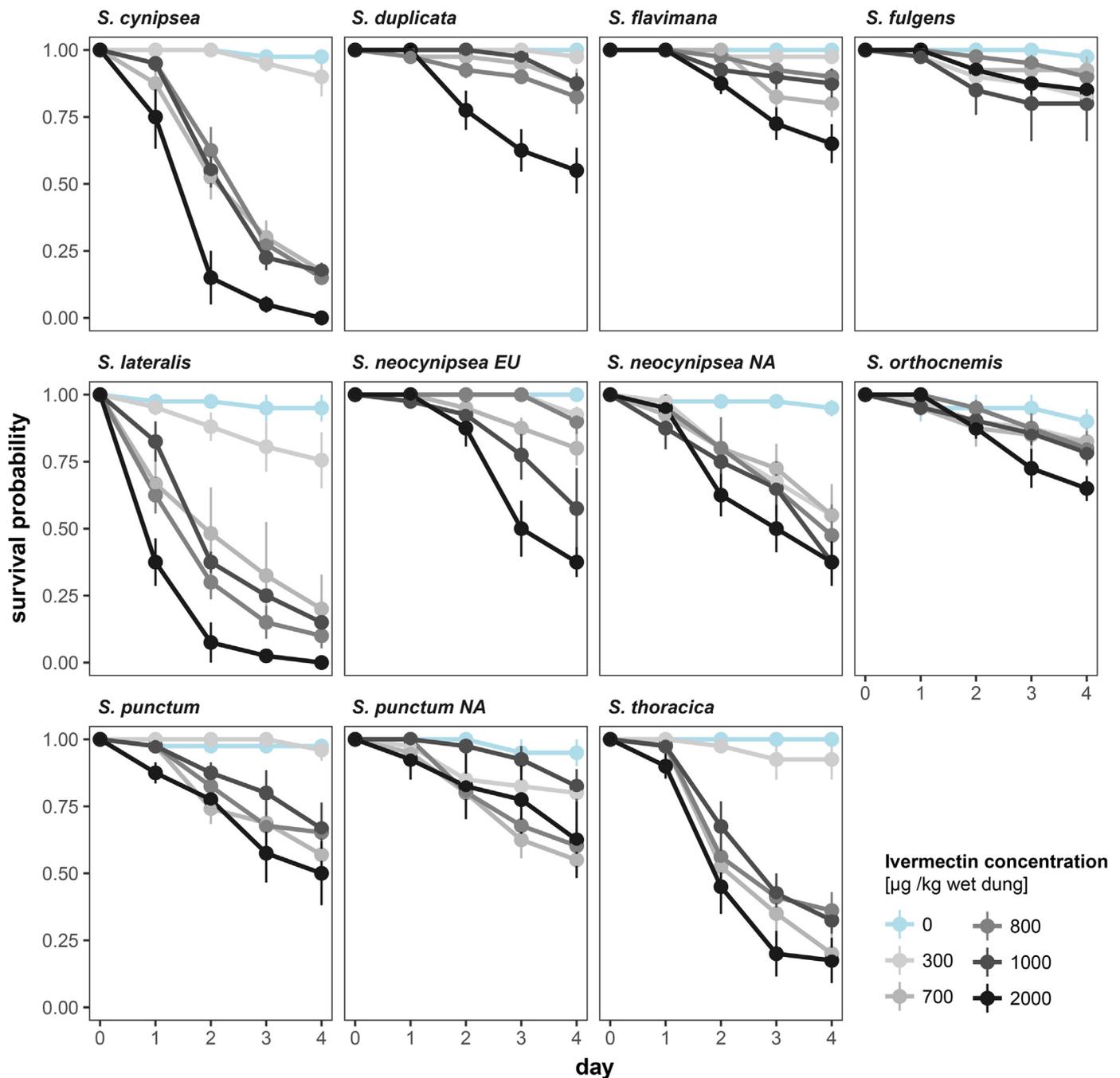


Fig. 1. Mean \pm SE proportion of survived flies across days for all eleven sepsid taxa as a function of adult ivermectin treatment ($n = 5$ replicates of 8 flies per treatment and taxon), analysed using Cox regression. All taxa except *S. fulgens* are negatively affected ($P < 0.05$) more or less strongly (taxon \times ivermectin treatment interaction: $X^2 = 40.06$; $P < 0.001$).

exposure (Table 3D). This effect was further amplified when both parents were exposed to ivermectin (treatment male \times treatment female interaction; Table 3D).

Exposure to ivermectin had no effect on offspring development time in our experiment, nor did it cause a change in the offspring sex ratio ($P > 0.2$). Also note that neither the number of emerged offspring nor survival improved during the five days the experiments was conducted (all day \times treatment interactions: $P > 0.13$).

3.3. Mating behaviour of *Sepsis punctum* males feeding on contaminated dung

Copulation frequency did not differ between males having been

exposed to acetone- (control) vs. ivermectin-contaminated dung (21 (75%) vs. 23 (65.7%) copulations; $X^2_1 = 0.272$, $P = 0.600$). Ivermectin also had no significant effect on copulation duration (median = 22.0, quartiles [17.5; 24.0] vs. 13.5 min [7.0; 29.5]); Mann-Whitney U -test; $Z = -1.21$; $P = 0.230$; $n = 44$). Mating latency (i.e. time from pair introduction to copulation) also did not differ between the two treatments (median = 8.0, quartiles [5.0; 12.0] vs. 5.5 min, [2.0; 19.0]; Mann-Whitney U -test; $Z = -0.77$; $P = 0.289$; $n = 43$).

3.4. Oviposition choice in the field

Confirming previous findings in the laboratory (Blanckenhorn et al., 2013), wild females were equally likely to lay their eggs into

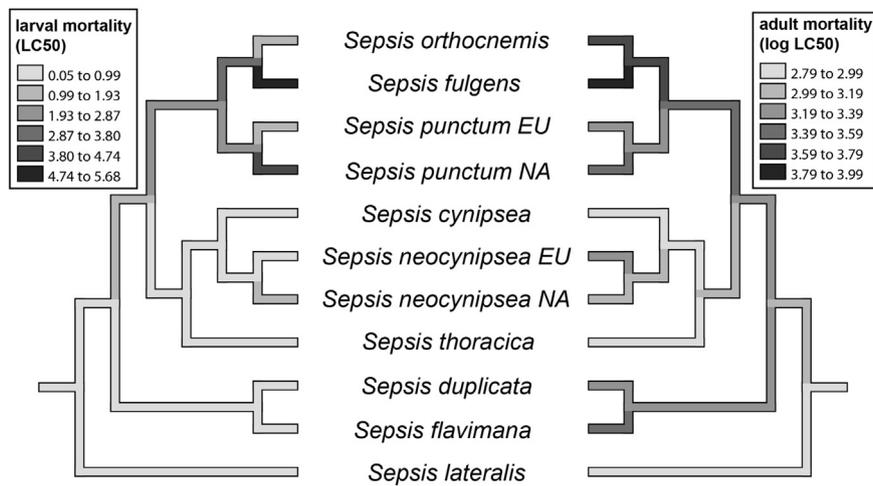


Fig. 2. Larval (left) and adult (right) ivermectin sensitivity (LC50: lethal concentration at which 50% of individuals die, in µg ivermectin/kg wet dung) mapped onto the phylogeny for eleven sepsid fly taxa.

ivermectin-spiked or control (acetone) dung, and hence are not able to detect or discriminate ivermectin in the field (Kruskal-Wallis-test: $P = 0.862$; $n = 10$; Supp. Fig. S1).

4. Discussion

Most research investigating the effects of ivermectin on dung organisms focuses on mortality during the juvenile (larval or pupal) stage, i.e. lethal effects (Lumaret et al., 2012). In contrast, by applying approaches from evolutionary biology and behavioural ecology, we here

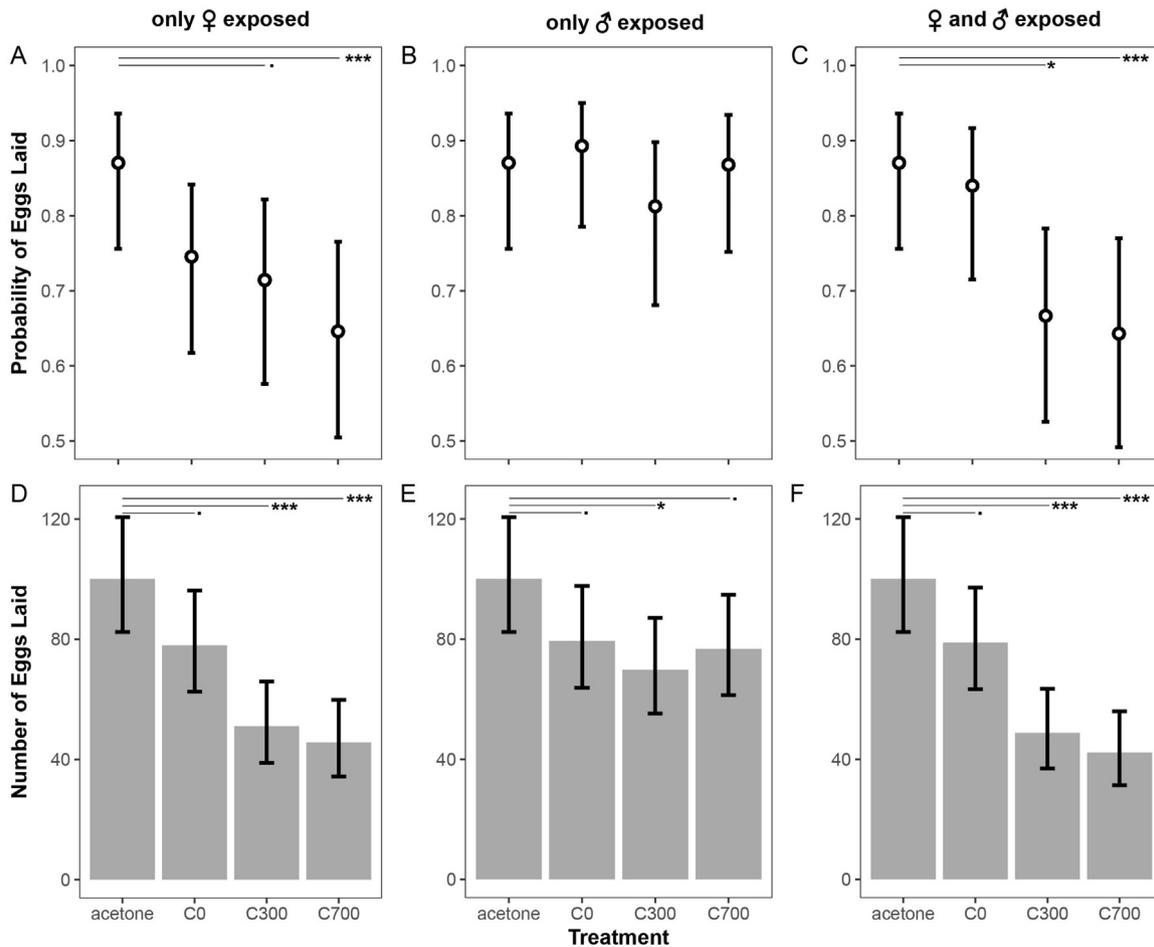


Fig. 3. Mean probability (top) and number (bottom) of eggs laid \pm 95%CI by *Sepsis punctum* when the female (A, D), the male (B, E) or both (C, F) were exposed to ivermectin of a given concentration ($n = 60$ pairs per treatment combination), analysed with General Linear Mixed Models. Female fecundity (A, C, D, F) but not male fertility (B, E) is gradually reduced as ivermectin concentration increases ($P < 0.001$).

Table 3
Analysis of variance (General Linear Mixed Models) for various fecundity traits of adult *Sepsis punctum* of both sexes feeding on ivermectin-contaminated dung (n = 60 pairs per treatment combination).

factor	A) p(eggs laid)			B) number of eggs laid		
	X ²	df	p	X ²	df	p
Female size	5.93	1	0.015	136.37	1	< 0.001
Male treatment	2.93	3	0.402	1.75	3	0.625
Female treatment	16.14	3	0.001	32.76	3	< 0.001

factor	C) p(offspring)			D) offspring number		
	X ²	df	p	X ²	df	p
Number of eggs	9.73	1	0.002	97.38	1	< 0.001
Male treatment	8.11	3	0.044	187.34	3	< 0.001
Female treatment	15.37	3	0.002	267.03	3	< 0.001
Male × female treatment				75.76	3	< 0.001

investigated the effects of ecologically relevant ivermectin concentrations (Liebig et al., 2010) on survival and reproduction of adult dung insects, many of which commonly lick dung even when merely using the substrate for oviposition (as opposed to food). We here demonstrated pronounced lethal as well as sublethal effects in terms of reduced adult longevity and fecundity. The increased mortality of adult sepsids broadly accords with the results of a previous study showing

that high ivermectin concentrations prompted greater larval mortality (Blanckenhorn et al., 2013). Moreover, the observed susceptibility of adult flies to ivermectin-contaminated dung varied among species in accordance with their larval susceptibility as shown by Puniamorthy et al. (2014), thus strengthening the presence of a strong (phylo)genetic signal (Fig. 2) and documenting systemic variation of ivermectin sensitivity in sepsids across life stages. However, the species-specific estimated LC50 values were three orders of magnitude (ca. thousand-fold) higher for adults than for larvae. Nevertheless, such high concentrations can occur in the field (Liebig et al., 2010), although adult sepsids are of course systemically exposed to contaminated dung for much briefer periods of time. Ivermectin disturbs ion transport through cell walls by binding to ion channels (Ōmura, 2008; Lumaret et al., 2012), thus impacting on non-target insect performance primarily in the phase of cell differentiation and growth and therefore making juvenile stages much more sensitive. In contrast, the mechanisms causing fitness reductions in adults remain unclear. The repeated and correlated demonstration of a systemic phylogenetic signal in the ivermectin sensitivity of juvenile and adult sepsid flies implies a minor role of environmental differences, e.g. in dung specialization, among species in explaining the variation found (cf. Pont and Meier, 2002). More importantly, it strengthens our previous interpretation (Puniamorthy et al., 2014) that presumably random evolution of ivermectin susceptibility by genetic drift, rather than natural selection for ivermectin tolerance, across the phylogeny (Fig. 2) preadapted (i.e. exapted) the comparatively resistant species of the *punctum* group to our modern, contaminated agricultural landscape.

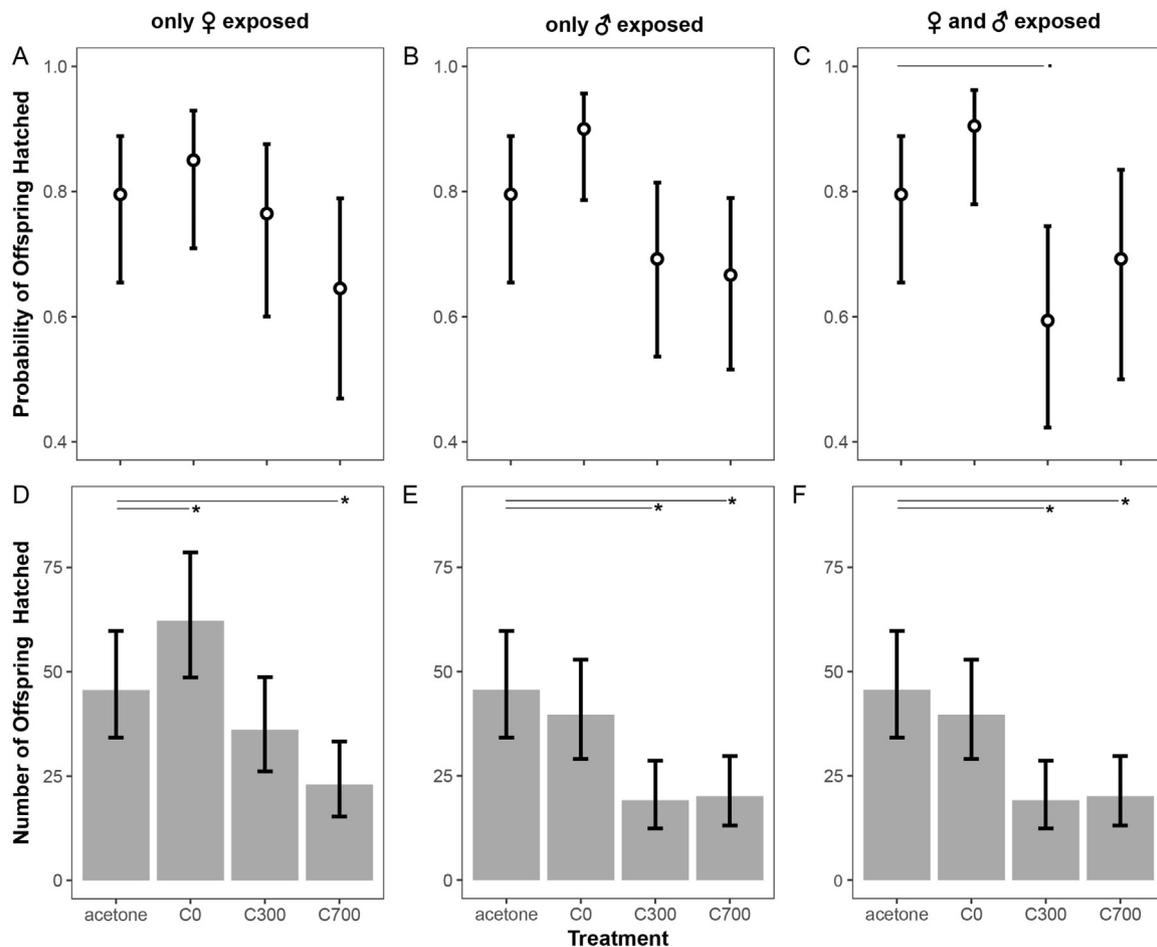


Fig. 4. Mean probability (top) and number (bottom) of emerged viable *Sepsis punctum* offspring \pm 95%CI when the female (A, D), the male (B, E) or both parents (C, F) were exposed to ivermectin of a given concentration (n = 60 pairs per treatment combination), analysed with General Linear Mixed Models. Female and male reproductive output are gradually reduced as ivermectin concentration increases ($P < 0.001$).

Numerous studies have shown that ivermectin application to livestock reduces the abundance and diversity of non-target organisms involved in dung degradation (Lumaret et al., 2012; see Floate et al., 2016 for a recent study). This can diminish pasture quality since lower insect activity in contaminated dung pats may delay dung degradation up to four-fold relative to untreated pats (Madsen et al., 1990; Floate, 1998; but see Tixier et al., 2016). Ecotoxicological research concluded that the detrimental effect of ivermectin is primarily caused by increased mortality of various, often beneficial dung insect species feeding on contaminated livestock faeces. Such larval feeding has also been reported to produce non-lethal effects in terms of prolonged development, suboptimal growth and/or stunted body size (e.g. in the yellow dung fly: Römbke et al., 2009; sepsids: Blanckenhorn et al., 2013), all ultimately reducing the performance and fitness of the emerging adult insects (Blanckenhorn, 2009). Our study suggests that the effects of ivermectin on the dung insect community may be even more far-reaching, as adult dung insects may additionally suffer from increased mortality if they themselves feed on contaminated dung.

Adult dung licking is obligatory in sepsids, as these scavenger flies so derive the necessary proteins for gamete production (Teuschl and Blanckenhorn, 2007), which are presumably derived from dung microorganisms or fungi. We here further found in one representative and relatively insensitive species, *S. punctum*, that adult feeding on dung contaminated with ivermectin reduced their reproductive success by impairing female fecundity (strongly) and, perhaps surprisingly, male fertility (weakly) via the quality and/or quantity of male sperm, documenting synergistic negative effects when both parents were exposed (Tables 2, 3). These fitness decrements of ivermectin are both direct, in that female egg and male sperm production are affected, but also indirect parental effects, because egg-to-adult survival of the produced offspring was also reduced. However, the physiological pathways mediating these effects are unclear and beg for further scrutiny. *Sepsis punctum* is relatively resistant (Figs. 1, 2), potentially contributing to their local abundance in landscapes dominated by animal husbandry. The fact that male copulatory behaviour in terms of mating frequency and copulation duration is not visibly compromised suggests that ivermectin acts at the level of sperm transfer and/or the production of sperm or seminal fluids. Other avermectins have also been found to impair the production of sperm in ticks and sperm motility in oysters (Montasser et al., 2005; Falkenberg et al., 2017). Our results corroborate these findings and suggest a general susceptibility of male gamete production to avermectins (cf. Lumaret et al., 2012). The reduction in female fecundity is likely caused by ivermectin directly influencing vitellogenesis, thereby leading to resorption of oocytes as recently reported for a dung beetle by Martinez et al. (2017), although reduced egg numbers might alternatively be explained by delayed egg development. Interestingly, even though fertility and fecundity were tested with uncontaminated dung, neither male nor female fitness noticeably recovered over a period of five days, suggesting that the effects of ivermectin uptake on gamete production might be long-lasting and possibly irreversible. Non-lethal ivermectin effects therefore likely have population-level consequences that may be further amplified by potential carry-over effects if contaminated adults disperse from one pasture to another. Ivermectin residues can have effects across trophic levels (e.g. on wasp parasitoids of sepsids: Floate, 1998; Jochmann and Blanckenhorn, 2016), such that local ivermectin application might disturb arthropod community structure at much broader spatial and ecological scales than currently assumed even though sepsids are small and poor flyers.

Gonzalez-Tokman et al. (2017b) similarly revealed lethal and sublethal effects of a herbicide on reproduction of the dung beetle *Euoniticellus intermedius* fed with contaminated dung. For the same species, Martinez et al. (2017) documented a fecundity reduction when exposed to ivermectin, which was caused by oocyte resorption following terminated vitellogenesis and abnormal ovary morphology. These authors further reported altered offspring sex ratios with female

larvae being more strongly affected and thus emerging at lower rates, which we here did not find for the offspring of *S. punctum* feeding on contaminated dung. Nor did we find parental effects on offspring development time. Whether all these effects are to be classified as direct or indirect parental effects depends on the life cycle of the dung organism. Several dung beetles form brood balls using the (contaminated) dung they were exposed to, into which an egg is laid for the hatched larva to feed on. Their offspring are therefore directly exposed to ivermectin, which in these species makes it difficult to separate indirect fitness consequences of parental exposure on their larvae from direct fitness consequences occurring through feeding on contaminated dung provided to the offspring by the parents. In our experiment with *S. punctum* only the parents were exposed to contaminated dung, which reduced the number of eggs laid by the mother and the number of offspring produced by both parents even when the juveniles developed in uncontaminated dung, a clear parental effect likely mediated by egg or sperm quality and/or quantity.

Despite visible detrimental effects of ivermectin on sepsid juveniles and adults, we found that ovipositing wild sepsid females (presumably primarily *S. punctum*) do not discriminate against ivermectin-contaminated dung, corroborating similar results previously obtained in the laboratory by Blanckenhorn et al. (2013), but contrasting attractive effects reported by Floate (2007). Other studies have revealed contrary evidence regarding the ability of various dung arthropods to perceive contaminants in the dung, so this question demands further study (Holter et al., 1993; Suarez et al., 2003; Webb et al., 2010; Lumaret et al., 2012). Conflicting results for sepsid flies suggest that the capacity to detect ivermectin (or, more generally, avermectins) may evolve in the presence of sufficient standing genetic variance, and if natural selection is strong and consistent enough across the landscape to produce changes in the flies' sensory system in the face of widespread parasiticide application over the past half century (Ōmura, 2008),

5. Conclusions

We here presented convincing evidence that ivermectin not only affects juvenile development and survival as shown in earlier publications (Iwasa et al., 2005; Blanckenhorn et al., 2013; Puniamoorthy et al., 2014), but that it has considerable additional fitness consequences when encountered by adult sepsid flies. Although intermediate concentrations of ivermectin are often not lethal, they nevertheless can have negative sublethal consequences for adult reproduction, particularly if the flies are unable to detect contamination. As these effects are not limited to the larval stage, they do not remain local, and through dispersal likely accumulate in the food chain via predation or other species interactions, with potentially far-reaching population-level ecological and evolutionary consequences. Sepsid flies are very abundant and widespread but rather small dung decomposers, hence of limited importance for dung degradation; and excessive ivermectin concentrations will typically be very localized to the excrements of few recently treated single livestock individuals on any given pasture. Nevertheless, further studies focussing on sublethal effects on other adult arthropods are needed to fully appreciate the ecological, evolutionary, and ultimately also the economic consequences of ivermectin use in livestock farming.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.07.029.

References

- Bates, D., Machler, M., Bolker, B.M., Walker, S.C., 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48.
- Blanckenhorn, W.U., 2009. Causes and consequences of phenotypic plasticity in body

- size: the case of the yellow dung fly *Scathophaga stercoraria* (Diptera: scathophagidae). In: Whitman, D.W., Ananthakrishnan, T.N. (Eds.), Phenotypic Plasticity of Insects: Mechanism and Consequences. Science Publishers, Enfield, NH, USA, pp. 369–422.
- Blanckenhorn, W.U., Puniamoorthy, N., Schafer, M.A., Scheffczyk, A., Rombke, J., 2013. Standardized laboratory tests with 21 species of temperate and tropical sepsid flies confirm their suitability as bioassays of pharmaceutical residues (ivermectin) in cattle dung. *Ecotoxicol. Environ. Saf.* 89, 21–28.
- Campbell, W.C., Fisher, M.H., Stapley, E.O., Albersschonberg, G., Jacob, T.A., 1983. Ivermectin – a potent new anti-parasitic agent. *Science* 221, 823–828.
- Desneux, N., Decourtye, A., Delpuech, J.M., 2007. The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* 52, 81–106.
- Falkenberg, L.J., Wrangle, A.L., Kinnby, A., Havenhand, J.N., Lockyer, A., Styan, C.A., 2017. Low sensitivity of reproductive life-stages in the Pacific oyster (*Crassostrea gigas*) to abamectin. *Chemosphere* 182, 665–671.
- Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.* 76, 122–159.
- Floate, K.D., 1998. Off-target effects of ivermectin on insects and on dung degradation in southern Alberta, Canada. *Bull. Entomol. Res.* 88, 25–35.
- Floate, K.D., 2007. Endectocide residues affect insect attraction to dung from treated cattle: implications for toxicity tests. *Med. Vet. Entomol.* 21, 312–322.
- Floate, K.D., During, R.A., Hanafi, J., Jud, P., Lahr, J., Lumaret, J.P., Scheffczyk, A., Tixier, T., Wohde, M., Rombke, J., Sautot, L., Blanckenhorn, W.U., 2016. Validation of a standard field test method in four countries to assess the toxicity of residues in dung of cattle treated with veterinary medical products. *Environ. Toxicol. Chem.* 35, 1934–1946.
- Gonzalez-Tokman, D., Martinez, M.I., Villalobos-Avalos, Y., Munguia-Steyer, R., Ortiz-Zayas, M.D., Cruz-Rosales, M., Lumaret, J.-P., 2017a. Ivermectin alters reproductive success, body condition and sexual trait expression in dung beetles. *Chemosphere* 178, 129–135.
- Gonzalez-Tokman, D., Martínez-Morales, I., Farrera, A., Ortiz-Zayas, M., Lumaret, J.-P., 2017b. Effects of a herbicide on physiology, morphology, and fitness of the dung beetle *Euoniticellus intermedius* (Coleoptera: Scarabaeidae). *Environ. Toxicol. Chem.* 36, 96e102.
- Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., de Kroon, H., 2017. More than 75% decline over 27 years in total flying insect biomass in protected areas. *PLoS One* 12, e0185809.
- Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.F., Aupinel, P., Aptel, J., Tchamitchian, S., Decourtye, A., 2012. A common pesticide decreases foraging success and survival in honey bees. *Science* 336, 348–350.
- Herd, R.P., Sams, R.A., Ashcraft, S.M., 1996. Persistence of ivermectin in plasma and faeces following treatment of cows with ivermectin sustained-release, pour-on or injectable formulations. *Int. J. Parasitol.* 26, 1087–1093.
- Holter, P., Sommer, C., Grønvald, J., 1993. Attractiveness of dung from ivermectin-treated cattle to Danish and afrotropical scarabaeid dung beetles. *Vet. Parasitol.* 48, 159–169.
- Iwasa, M., Nakamura, T., Fukaki, K., Yamashita, N., 2005. Nontarget effects of ivermectin on coprophagous insects in Japan. *Environ. Entomol.* 34, 1485–1492.
- Jochmann, R., Blanckenhorn, W.U., 2016. Non-target effects of ivermectin on trophic groups of the cow dung insect community replicated across an agricultural landscape. *Basic Appl. Ecol.* 17, 1947–1952.
- Liebig, M., Fernandez, A.A., Blubaum-Gronau, E., Boxall, A., Brinke, M., Carbonell, G., Egeler, P., Fenner, K., Fernandez, C., Fink, G., Garric, J., Halling-Sørensen, B., Knacker, T., Krogh, K.A., Kuster, A., Löffler, D., Cots, M.A.P., Pope, L., Prasse, C., Römcke, J., Ronnefahrt, I., Schneider, M.K., Schweitzer, N., Tarazona, J.V., Ternes, T.A., Traunspurger, W., Wehrhan, A., Duis, K., 2010. Environmental risk assessment of ivermectin: a case study. *Integr. Environ. Assess. Manag.* 6 (Suppl.), S567–S587.
- Lu, Y.H., Wu, K.M., Jiang, Y.Y., Guo, Y.Y., Desneux, N., 2012. Widespread adoption of Bt cotton and insecticide decrease promotes biocontrol services. *Nature* 487, 362–365.
- Lumaret, J.P., Errouissi, F., Floate, K., Rombke, J., Wardhaugh, K., 2012. A review of the toxicity and non-target effects of macrocyclic lactones in terrestrial and aquatic environments. *Curr. Pharm. Biotechnol.* 13, 1004–1060.
- Madsen, M., Nielsen, B.O., Holter, P., Pedersen, O.C., Jespersen, J.B., Jensen, K.M.V., Nansen, P., Grønvald, J., 1990. Treating cattle with ivermectin – effects on the fauna and decomposition of dung pats. *J. Appl. Ecol.* 27, 1–15.
- Martin, O.Y., Hosken, D.J., 2002. Strategic ejaculation in the common dung fly *Sepsis cynipsea*. *Anim. Behav.* 63, 541–546.
- Martinez, M.I., Lumaret, J.P., Zayas, R.O., Kadiri, N., 2017. The effects of sublethal and lethal doses of ivermectin on the reproductive physiology and larval development of the dung beetle *Euoniticellus intermedius* (Coleoptera: scarabaeidae). *Can. Entomol.* 149, 461–472.
- Montasser, A.A., Gadelhak, G.G., Tariq, S., 2005. Impact of ivermectin on the ultra-structure of the testis of *Argas (Persicargas) persicus* (Ixodoidea: argasidae). *Exp. Appl. Acarol.* 36, 119–129.
- Ömura, S., 2008. Ivermectin: 25 years and still going strong. *Int. J. Antimicrob. Agents* 31, 91–98.
- Orme, D., Freckleton, R., Thomas, G., Petzoldt, T., Fritz, S., Isaac, N., Pearce, W., 2013. Caper: Comparative analyses of phylogenetics and evolution in R. Collingwood. CSIRO Publishing, Australia.
- Pont, A.C., Meier, R., 2002. The Sepsidae (Diptera) of Europe. *fauna. Entomol. Scand.* 37, 1–221.
- Puniamoorthy, N., Schafer, M.A., Blanckenhorn, W.U., 2012. Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly *Sepsis punctum* (Diptera: sepsidae). *Evolution* 66, 2117–2126.
- Puniamoorthy, N., Schafer, M.A., Rombke, J., Meier, R., Blanckenhorn, W.U., 2014. Ivermectin sensitivity is an ancient trait affecting all ecdysozoa but shows phylogenetic clustering among sepsid flies. *Evolut. Appl.* 7, 548–554.
- R Development Core Team, 2008. R: A language and environment for statistical computing. Vienna, Austria.
- Rohner, P.T., Blanckenhorn, W.U., Puniamoorthy, N., 2016. Sexual selection on male size drives the evolution of male-biased sexual size dimorphism via the prolongation of male development. *Evolution* 70, 1189–1199.
- Rohner, P.T., Teder, T., Esperk, T., Lüpold, S., Blanckenhorn, W.U., 2018. The evolution of male-biased sexual size dimorphism is associated with increased body size plasticity in males. *Funct. Ecol.* 32. <https://doi.org/10.1111/1365-2435.13004>.
- Römcke, J., Floate, K.D., Jochmann, R., Schafer, M.A., Puniamoorthy, N., Knabe, S., Lehms, J., Rosenkranz, B., Scheffczyk, A., Schmidt, T., Sharples, A., Blanckenhorn, W.U., 2009. Lethal and sublethal toxic effects of a test chemical (Ivermectin) on the yellow dung fly (*Scathophaga stercoraria*) based on a standardized international ring test. *Environ. Toxicol. Chem.* 28, 2117–2124.
- Schaeffer, M., Turner, M.J., 1989. Ivermectin and Abamectin. Springer, New York.
- Schmitt, H., Römcke, J., 2008. The ecotoxicological effects of pharmaceuticals (antibiotics and antiparasitics) in the terrestrial environment – a review. In: *Pharmaceuticals in the Environment*. Springer, Heidelberg, pp. 285–303.
- Skidmore, P., 1991. Insects of the British Cow-dung Community. Field Studies Council, Montford Bridge, Shrewsbury.
- Suarez, V.H., Lifschitz, A.L., Sallovitz, J.M., Lanusse, C.E., 2003. Effects of ivermectin and doramectin faecal residues on the invertebrate colonization of cattle dung. *J. Appl. Entomol.* 127, 481–488.
- TerHorst, C.P., Lau, J.A., Cooper, I.A., Keller, K.R., La Rosa, R.J., Royer, A.M., Schultheis, E.H., Suwa, T., Conner, J.K., 2015. Quantifying nonadditive selection caused by indirect ecological effects. *Ecology* 96, 2360–2369.
- Teuschl, Y., Blanckenhorn, W.U., 2007. The reluctant fly: what makes *Sepsis cynipsea* females willing to copulate? *Anim. Behav.* 73, 85–97.
- Therneau, T.M., Grambsch, P.M., 2013. *Modeling Survival Data: Extending the Cox Model*. Springer, New York.
- Tixier, T., Blanckenhorn, W.U., Lahr, J., Floate, K., Scheffczyk, A., During, R.A., Wohde, M., Rombke, J., Lumaret, J.P., 2016. A four-country ring test of nontarget effects of ivermectin residues on the function of coprophilous communities of arthropods in breaking down livestock dung. *Environ. Toxicol. Chem.* 35, 1953–1958.
- Webb, L., Beaumont, D.J., Nager, R.G., McCracken, D.I., 2010. Field-scale dispersal of *Aphodius* dung beetles (Coleoptera: scarabaeidae) in response to avermectin treatments on pastured cattle. *Bull. Entomol. Res.* 100, 175–183.
- Zhang, W., Ricketts, T.H., Kremen, C., Carney, K., Swinton, S.M., 2007. Ecosystem services and dis-services to agriculture. *Ecol. Econ.* 64, 253–260.
- Zhao, L., Ang, S.H.A., Srivathsan, A., Su, K.F.Y., Meier, R., 2013. Does better taxon sampling help? A new phylogenetic hypothesis for Sepsidae (Diptera: cyclorhapha) based on 50 new taxa and the same old mitochondrial and nuclear markers. *Mol. Phylogenet. Evol.* 69, 153–164.