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# The veterinary drug ivermectin influences immune response in the yellow dung fly (*Scathophaga stercoraria*)

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*Phenoloxidase activity in Scathophaga stercoraria is enhanced by ivermectin and that effect is transferred to the adult fly from the larval stage.*

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ABSTRACT

Phenoloxidase (PO) is a key enzyme involved in the immune response of insects. We show that egg-to-adult exposure to residues of 0.001, but not 0.0005 mg $kg^{-1}$ ivermectin elevated PO activity in yellow dung flies (*Scathophaga stercoraria*) developing in cattle dung. Fly fat content was unaffected by the treatments. Therefore, the response of PO was a direct effect of ivermectin and not an indirect one caused by an alteration in body 'condition'. This supports the non-intuitive conclusion that flies surviving exposure to faecal residues may have enhanced immune function. To our knowledge, this is the first study to assess the effects on PO activity of insecticidal residues in livestock dung. The non-target effects of such residues are of wide interest, given the global use of veterinary products.



## 1. Introduction

Ivermectin (22,23-dihydroavermectin) is a synthetic derivative of the macrocyclic lactone abamectin. It has broad-spectrum activity against arthropods and nematodes (Campbell, 1985) and is routinely administered to livestock. Following treatment, ivermectin is released into the environment *via* the faeces; the amount released varies according to route of administration (Lumaret and Errouissi, 2002). When injected or given as a topical pour-on, elimination mainly occurs within 2–7 days, with smaller amounts still excreted up to 6 weeks after treatment. Residues may be detected for up to 147 days after sustained-release bolus application (see Floate et al., 2005). The potentially detrimental effects of ivermectin on non-target invertebrates have been widely reported (e.g. McCracken and Foster, 1993; Suarez et al., 2003; Floate et al., 2005) and range from acute toxicity leading to death, to chronic sub-lethal toxicity resulting in delayed development and physiological abnormalities (Strong and James, 1993; Krüger and Scholtz, 1995).

The yellow dung fly (*Scathophaga stercoraria*) is a common species of dung-breeding fly whose immune function has been extensively studied (Hosken, 2001; Schwarzenbach et al., 2005; Schwarzenbach and Ward, 2006, 2007). Insect immunity consists of a variety of infection-mediated responses (Schmid-Hempel, 2005), one of which is the melanisation process (Söderhäll and Cerenius, 1998). Foreign particles are encapsulated in a melanised deposit as a result of the enzyme phenoloxidase (PO). PO exists in the haemolymph as an inactive form (proPO) which is converted into active PO by invasion of foreign materials. PO activity is often used as a measure of potential immunity (see Schmid-Hempel, 2005).

The effect of heavy metal pollution on the immune response of ant and moth species has recently received attention (Sorvari et al., 2007; Van Ooik et al., 2007); these studies showed that immune defence was enhanced by moderate metal pollution, although in ants, high levels of pollution suppressed the response.

This current investigation is the first study to consider the effect of veterinary drug residues on insect immune response. The aim was to measure PO activity in yellow dung flies originating from larvae reared in dung amended with sub-lethal concentrations of ivermectin and in unamended dung.

## 2. Materials and methods

### 2.1. Experimental set-up

Fresh dung was collected from an ivermectin-free, organic herd of cattle in Leicestershire, UK and assigned to one of 3 treatments: (1) control; (2) 0.0005 mg $kg^{-1}$ ivermectin; (3) 0.001 mg $kg^{-1}$ ivermectin on a dry weight basis. This equated to 0.0001 mg $kg^{-1}$ and 0.0002 mg $kg^{-1}$ on a fresh weight basis. These low concentrations were selected following preliminary studies and aimed to minimise overt developmental abnormalities observed by Strong and James (1993) at higher levels. Ivermectin solutions were prepared by dissolving ivermectin (Sigma, Poole, UK) in ethanol and 2 mL aliquots of the required concentration were added to 0.5 kg

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wet weight quantities of dung and mixed thoroughly for 15 min. Ethanol (2 mL) was applied to the same quantity of dung for the control treatment and mixed thoroughly. Dung was left overnight so that residual ethanol would be removed before transferring to individual experimental units. Freshly laid eggs were collected from the surface of dung pats produced by an ivermectin-free, organic herd of cattle. Approximately 500 eggs were allowed to hatch in dung of the three treatments and on hatching, active larvae were transferred to experimental vials containing the corresponding concentration of ivermectin. Each larva had access to 2.5 g of dung (Amano, 1983). The experimental unit was the individual. Egg, larval, pupal and adult stages were maintained in a climate chamber (19–20 °C; 12L:12D photoperiod) and randomly positioned within the chamber. Within 48 h after emergence, flies were anaesthetised with $CO_2$ and frozen at −80 °C until analysed.

### 2.2. Body mass and fat content

All assays were conducted on newly emerged male and female flies that had been kept individually, were not sexually mature and had not been fed. Body mass and fat content were measured on a sub-set of the flies (15 female and 15 males flies per treatment; chosen randomly). Flies were dried (24 h at 55 °C) and weighed. Dried flies were left individually in 5 mL ether for 24 h, after which they were dried again at 55 °C for 3 h and re-weighed. The weight difference is the fat content of the fly (method modified from Sundström, 1995).

### 2.3. PO activity and general protein

The remaining flies (not used in Section 2.2 above) each had their wings and legs removed on ice and their bodies were homogenised in 60 μL ice cold sodium cacodylate buffer (0.01 M NaCac, 0.005 M CaCl; pH 6.5; from Sigma) and centrifuged at 16,233*g* for 10 min at 4 °C to obtain the haemolymph. Forty microliters of the supernatant were collected and immediately frozen (−80 °C) until required. The haemolymph was used for both PO and protein assays. PO assays were conducted using a modification of the method described by Schwarzenbach et al. (2005). Into individual wells of a microtitre plate, 5 μL of the haemolymph was added to 93 μL Milli-Q water and 12.0 μL of PBS (8.74 g NaCl, 1.78 g $NaH_2PO_4 \cdot H_2O$ in 1 L Milli-Q $H_2O$; pH 6.5). Then 15 μL of L-Dopa (4 mg $mL^{-1}$ Milli-Q $H_2O$) was added and the solution assayed by reading absorbance at 480 nm every 60 s for 60 min using a microplate reader (Bio-Rad Model 3550, Hemel Hempstead, UK). PO activity was determined from the slope of the reaction (absorbance vs. time) during the linear phase of the reaction, which was generally between 5 and 20 min from the start of the reaction. Absorbance was corrected against control wells containing Milli-Q water instead of haemolymph.

Protein concentration was measured using Bradford reagent (Sigma). Briefly, 5 μL of haemolymph was added to 45 μL NaCac buffer and 200 μL Bradford reagent in microtitre plates, incubated for 15 min and read at 595 nm using a microplate reader. Standards were made from bovine albumin (Sigma).

For all flies that had PO activity and protein concentration measured, hind tibia lengths were measured using a binocular microscope and the average of the left and right side used in statistical analyses.

### 2.4. Statistical analyses

Data were analysed by either analysis of variance or analysis of covariance using GenStat Release 8.1 (Lawes Agricultural Trust). Data were $\log_{10}$ transformed where appropriate if not normally distributed. To remove negative values some transformations were $\log_{10}(+1)$ or $\log_{10}(+4)$ (for protein and PO data, respectively). Normality was tested by plotting residuals against expected normal quantiles in GenStat. Only those plots producing a linear relationship were accepted as normal. Further, plots of residuals against fitted values were used to check for any relationship between them which would invalidate the assumption of independence of errors and fitted values. Analysis of variance was performed on body mass, percentage fat content and tibia length data, using ivermectin concentration and sex as factors. Analysis of covariance was performed on protein concentration and on PO activity data using tibia length (for both variables) and general protein content (for PO analysis) as covariates.

## 3. Results

Ivermectin exerted some lethal effects. Of the ivermectin-treated larvae, 28% of those treated with 0.0002 mg $kg^{-1}$ and 44% subjected to 0.0001 mg $kg^{-1}$ (fresh weight basis) successfully pupated; 91% of the controls pupated. Flies eclosed from an overall mean of 63% of the pupae, irrespective of treatment. Therefore, treatment with ivermectin did not influence the number of flies that successfully eclosed, only the number of larvae that pupated. The treatment-related mortality meant that the following numbers of replicates were used in analyses: 30 of each treatment for fat and dry weight measurements; 80 control flies and 80 and 60 ivermectin-treated flies (0.0001 and 0.0002 mg $kg^{-1}$, respectively) for PO, protein and tibia length measurements. This left a number of 'spare' control flies that were used for method development.

### 3.1. Hind tibia lengths and fat content

Mean hind tibia lengths were significantly longer in males than in females (males = 3.01 mm and females = 2.46 mm, $F_{1,202} = 180.97$, $p < 0.001$) indicating that females were smaller than the males. This corroborates the dry weight data obtained for a smaller sample of flies that were weighed prior to fat analysis (females = 1.17 mg and males = 2.19 mg; ANOVA for dry weight as a single factor, $p = 0.001$). The percentage of fat content of flies was unaffected by ivermectin amendment of dung or by sex (overall mean = 6.58% fat), although fat content (mg) was related to body mass, with larger flies containing more fat (data not shown).

### 3.2. Phenoloxidase activity and protein content

Phenoloxidase (PO) reaction rate was significantly increased in flies that had been reared in dung amended with the highest level of ivermectin, but not in flies reared in dung containing the lower concentration of ivermectin (Fig. 1A). Tibia length was included as a covariate to prevent size differences from potentially obscuring the effects of ivermectin. The covariate was not significant ($F_{1,201} = 0.91$, ns). PO reaction rate was similar in both sexes (sex, $F_{1,201} = 0.07$, ns). Protein concentration was also included as a covariate in a separate analysis in order to establish whether ivermectin specifically affected PO, or if enhanced PO activity was a result of increased protein levels. Protein level as a covariate was marginally significant ($F_{1,192} = 3.5$, $p = 0.063$). Adjusted means for

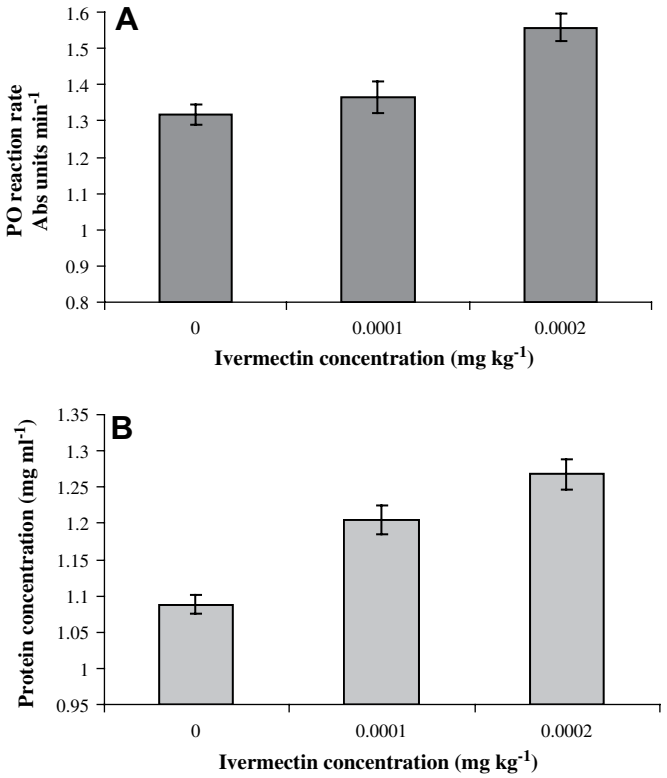


**Fig. 1.** (A) Activity of phenoloxidase (absorbance units $min^{-1}$) over the linear phase of the reaction in *Scathophaga stercoraria*. Data are $\log_{10}(+4)$ transformed means (ivermectin treatment as a single factor, $F_{2,201} = 18.44$, $p < 0.001$). Ivermectin concentrations are expressed on a fresh weight basis. (B) General protein content of haemolymph of *S. stercoraria*. Data are $\log_{10}(+1)$ transformed means (ivermectin treatment as a single factor, $F_{2,188} = 32.73$, $p < 0.001$).