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Nitrates and Herbicides Cause Higher Mortality than the Traditional Organic Fertilizers on the Grain Beetle, *Tenebrio molitor*

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Abstract We examined experimentally whether fertilizers or herbicides commonly used by farmers affect mortality of the adult grain beetle *Tenebrio molitor*. After a period of 4 weeks in direct contact with all treatments, a higher percentage of mortality occurred in contact with nitrates than with pig manure or turkey litter. Herbicides (a mixture of glyphosate and 2,4-D) caused 100% mortality. Our results also indicate that more beetles escaped from the herbicides and nitrate treatments than from the others, suggesting some kind of behavioural avoidance of toxic environments. The traditional organic fertilizers appear to be less toxic than inorganic fertilizers for *Tenebrio molitor*.

Keywords Yellow mealworm · Glyphosate · 2,4-D · Nitrates · Farming management · Insect conservation · Beetle behavior · Wildlife conservation

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Changes in modern agricultural practice have allowed an increased production through the use of fertilizers, herbicides and other pesticides. In the second half of the twentieth century many zones in the world have doubled the use of inorganic nitrogen, causing important negative changes for the wild flora and fauna (Vickery et al. 2001). Poultry (e.g., broiler, turkey) litter is extensively applied to agricultural lands as organic fertilizer because it contains high levels of elements that favour plant growth (Ponder et al. 2005). However, trace elements (e.g. copper and zinc) are often added to poultry diets to increase resistance to diseases, and therefore the litter also contains a large variety of trace elements which are potentially toxic to living systems when present in sufficient concentrations (Kpomblecou et al. 2002). Also other organic fertilizers such as pig manure can be toxic due to high concentrations of arsenic (Li and Chen 2005). Organic fertilizers contain bacteria such as *E. coli* and *Salmonella* sp (Castilla et al. 2008), and can also have a large variety of microbial communities and parasites that can be rather pathogenic (Bagge et al. 2009).

In many regions of Spain where farming activity is high and constitutes an important economical source for the human population, the use of organic fertilizers such as manure from animal farms is intense. They are used to increase cereal growth, but occasionally manure from animal farms is also discarded on fields. In many cases farmers eliminate much larger quantities of organic fertilizers than are allowed by law. This poses a considerable threat to many species. Fertilizers and agrochemicals also contain toxic heavy metals that have been found in the soils of agricultural fields in Spain (Adrover et al. 2007 and references cited).

In a previous study we examined the effect of commonly used mixtures of organic and mineral fertilizers, and herbicides on the mortality of larvae of the yellow mealworm (*Tenebrio molitor*) (Castilla et al. 2008). For the present study we produced in our laboratory adult beetles (*Tenebrio molitor*) of the same age to compared rates of mortality under different pesticide treatments.

Materials and Methods

We tested two types of organic fertilizers (turkey and pig manure), one mineral fertilizer, and one herbicide mixture, commonly used in farming practices in Catalonia. The turkey manure came from 2 month old birds that were not medicated at that time and was obtained from a local farm. The pig manure was obtained from young and adult animals that were medicated against the itch infection. At the time of the study, we used the available products that were being applied in the field, despite the differences in medication. Unfortunately, a detailed analysis of both organic fertilizers was not available.

We used a mixture of two types of synthetic herbicides (1 L of the isopropylamine salt of Glyphosate (Logrado, Masso Division Agro), 36% p/v (360 g/L), and 100 cm^3 of 2,4-D (Agrodan), 80%, in 4 L of water), both herbicides commonly used in the zone, were also examined. Using a binary mixture makes it difficult to deduce the individual effect of each herbicide to the insect. However, in our study we wanted to know the effect of the herbicides actually used in the field in our study area on *T. molitor* mortality. The farmers there mix two types of herbicides and apply such mixture in the field.

The mineral fertilizer consisted of dry nitrate pellets dissolved in water (27%). The analysis of the turkey excrements used in this study showed very high levels of pathogenic microorganisms, and comprises (*Escherichia coli* = 3,300,000 UFC/g, aerobic bacteria = 480,000,000 UFC/g, anaerobic bacteria: *Clostridium perfringens* = 13,000 UFC/g, *Salmonella* = positive, mucor fungi with filaments = 700,000 colonies/g, and yeast = 15,000,000 colonies/g).

The insect *Tenebrio molitor* was chosen as an assay animal because the larvae are commercially available and very easy to reproduce in captivity. At the Biological Station of Sanaüja we produced adult beetles of the same age (2-week-old) and size (mean mass = 1.10 g, sd = 0.05, n = 180). Beetles were placed in manufactured soft aluminium open boxes (16 × 11 × 3 cm). A set of four boxes were employed for each of the five treatments (Table 1) (9 individuals in each box; total = 36 beetles per treatment, and a total of 180 individuals). During the experiments many beetles escaped from the boxes, particularly from some treatments. That behaviour was unexpected since adults did not escape during preliminary observations under control conditions (with no toxic products available). We thus repeated the experiments by covering the boxes with sealing film and made several holes for respiration. A set of 3 boxes were employed for each treatment (6 individuals in each; total = 18 beetles per treatment, and a total of 90 individuals).

All boxes were kept under similar artificial environmental conditions (dark room with a mean temperature of 25°C, range: 24–26°C), at the Agriculture School of Solsona (Catalonia, Spain). Beetles in all treatments were fed with similar type and quantity of food: 12 g of white wheat, 3 g of dry powdered cat food, 10 g of green lettuce and ca. 10 g slice of white bread. A piece of cellulose paper (10 × 8 cm) was also supplied. Water (10 mL) was added once a week to each slice of bread.

The frequencies of beetle mortality in the covered boxes and the frequencies of beetle escape in the not-covered boxes, have been tested with Chi square test (one-by-one comparisons), using SPSS V. 15.

Results and Discussion

During a period of 4 weeks in the not-covered boxes, all beetles died in the herbicide (100%, 15 of 15 individuals) and half of them (50%, 3 of 6) in the nitrate treatment. However, none of the beetles died in contact with turkey excrements or pig manure, and none of them died in the control (Table 1). Many beetles escaped from the non-covered boxes (Table 1), particularly from the nitrate (83%, 30 of 36) and the herbicide (58%, 21 of 36) treatments (Table 1), and the proportions were significantly higher than those for the control treatment ($p < 0.001$ in both cases) or the other treatments (Table 2).

The fact that many beetles escaped altered our experimental design. However, the data still show clear differences in mortality rates among treatments. In addition, the results of the second experiment performed in covered boxes where the beetles could not escape, showed the same trend of higher mortality in the herbicide (100%, 18 of 18) and nitrate (72%, 13 of 18) treatments (Table 2). In this second experiment, mortality was present in all treatments including the control (6%, 1 of 18). Mortality rate in the turkey treatment was of 33% (6 of 18) and in pig manure of 44% (8 of 18) (Table 2).

Table 1 Number (n) and percentage (%) of beetle mortality in different types of boxes and treatments, after a period of 4 weeks

	Not-covered boxes (initial sample of 36 adults/treatment)				Covered boxes (initial sample of 18 adults/treatment)		
	n (escaped)	n (remaining)	n (dead, 4 weeks)	% Mortality 4 weeks	n	n (dead, 4 weeks)	% Mortality 4 weeks
Control	6	30	0	0	18	1	6
Nitrates	30	6	3	50	18	13	72
Herbicides	21	15	15	100	18	18	100
Turkey excrements	15	21	0	0	18	6	33
Pig excrements	3	33	0	0	18	8	44

In the not-covered boxes the initial number of beetles and those remaining after escape behaviour is also indicated

Table 2 One-by-one comparisons (Chi square test) of the percentage of adult mortality in the covered boxes, and of adults that escaped from their boxes under different treatments after 4 weeks

	Adult mortality in covered boxes		Adults escaped in not-covered boxes	
	Chi square	*p*	Chi square	*p*
C vs N	16.8	0.0000	32.0	0.0000
C vs H	32.2	0.0000	13.3	0.0003
C vs T	4.43	0.035	5.45	0.019
C vs P	7.26	0.007	1.14	0.3
N vs H	5.81	0.016	5.45	0.020
N vs T	7.4	0.0007	13.3	0.0003
N vs P	2.86	0.09	40.8	0.0000
H vs T	20.9	0.0000	2.00	0.16
H vs P	13.9	0.0002	20.25	0.0000
T vs P	0.47	0.5	10.7	0.0011

C control, *N* nitrates, *H* herbicides, *T* turkey excrements, *P* pig manure

We did not find any abnormal appearance in the beetles after being exposed to all different treatments in both experiments. Beetle survivors in the not-covered boxes produced fertile eggs and juveniles that survived at least 1 month. We did not check the beetles in the covered boxes after 4 weeks.

Our study, although has been conducted with a small sample size, it demonstrates that different products which are commonly used in agricultural practices are noxious for the beetle *Tenebrio molitor*, and the larvae of the same species (Castilla et al. 2008), particularly the herbicides and nitrates.

The effect of the products employed appeared to be different depending on the exposure conditions (i.e., open or close boxes). The microclimatic conditions (humidity and temperature) and the volatile particles of each treatment condensed inside the covered boxes appeared to have an additional influence on beetle mortality. The strong negative effects that herbicides and other pesticides have on wildlife have already been demonstrated (Lizotte et al. 2009). Also, bird excrements from aviaries can be dangerous for wildlife, as they may contain fungi, bacteria and small insects. It has been shown that *T. molitor* can be parasitized by many species of invertebrates (Warr et al. 2004) including those found in poultry manure. In many barns with infected ruminants, *Mycobacterium avium* has been isolated, and *T. molitor* can be infected with these bacteria in naturally contaminated bran and peat (Fischer et al. 2004). Consequently, infected beetles could mechanically transmit mycobacteria to their predators, that could also be infected, and increase the chain of diseases.

Because the percentages of mortality in all treatments were higher for larvae (Castilla et al. 2008) than for adult *T. molitor* (present study), our results suggest that the resistance to agrochemicals may increase with age, and that adults could behaviourally reduce the negative effect of those products by not consuming them or avoiding them. Other authors have also indicated that the effect of pesticides on insects depends on their developmental stage (Kostaropoulus et al. 2001).

Many beetles exposed to herbicides and nitrates escaped from their boxes. That was surprising, because in preliminary experiments the beetles did not escape from the control boxes. Thus, it appears that the worst treatments could have invoked this behaviour. In other studies, *T. molitor* has also shown behavioural avoidance to toxic compounds (García et al. 2003), and some beetles and larvae avoid feeding on herbicide-treated plants (Piesik and Lamparski 2004). Thus, the results of our study suggest that *T. molitor* adults could have developed some mechanisms to escape from inadequate environments.

Preliminary observations also showed that the development of pupae during the first week was very low and only started in nitrates and herbicides. Thus, another response to harmful environments could be an accelerating development.

Studies conducted with other organisms (plants and vertebrates) have shown the advantages of early emergence in seeds in poor environments (Verdú and Traveset 2005), and the adaptive developmental plasticity in amphibians or their local adaptations to stressful environments (Denver et al. 1998; Gomez-Mestre and Tejedo 2003).

The effect of farming on biodiversity is a considerable threat to many organisms, including plants, insects and birds (Moreby and Southway 1999). The present study and a previous one (Castilla et al. 2008) have shown that most products used in agriculture in our study area are toxic to *Tenebrio molitor* under different developmental stages. Although we only examined the response of one species to those products, it is rather likely that other species of the same genus will be similarly affected. Tenebrionids are the most preferred food items for many farmland species, and the decrease in invertebrates associated with farming practices affects the survival and successful reproduction of birds (Hart et al. 2006), lizards and other species.

Manure can contain bacterial spores and possibly also vegetative bacteria that pass though the digestive systems of animals grazing on contaminated pasture, and spore-forming bacteria may survive pasteurisation and digestion (Bagge et al. 2009 and references cited). Thus, to reduce the risk of spreading pathogens, the recommended bio-waste treatment method before anaerobic digestion is heating to 70°C for 1 h if manure and animal by-products are present in the substrate, as regulated by EU Commission regulation EC n° 1774/2002 and 208/2006 (Bagge et al. 2009). Heating at 70°C for 1 h reduces *Salmonella spp* (Larsen 1995), however, other spore-forming bacteria (e.g., *Clostridium, Bacillus*) are nor eradicated (references in Bagge et al. 2009).

Because agrochemical products negatively affect biodiversity, they should be spread with caution and adequately treated before being dispersed in the field. Several authors have suggested that management to conserve and increase animal number and species should concentrate on improving foraging habitat quality (Browne et al. 2006). Thus, a higher control of agricultural practices should be conducted by the local administrations, and more funding of projects focused on the relationships between farming and biodiversity should be encouraged.

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