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Sensitivity of preparasitic stages of *Chordodes nobilii* (Gordiida, Nematomorpha) to malathion

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Abstract The largest part of the life cycle of Gordiida, known as horsehair worms, occurs in aquatic environments usually affected by agricultural activities. The free-living adults reproduce in freshwater environments, where preparasitic larvae undergo development. Since malathion is an insecticide used in the distribution area of *Chordodes nobilii*, the aim of this study is to evaluate the effect of malathion concentrations which might be expected in the environment on preparasitic stages of this species. The embryonic development and the viability of larvae were analyzed after a short-term exposure to malathion concentrations ranging between 36 and 220 μg a.i./l. Embryo development was inhibited at 220 μg a.i./l and the infective capacity of larvae derived from malathion-exposed eggs was significantly decreased starting from the lowest concentration. Larvae developed from malathion-exposed eggs exhibited malformations. Directly exposed larvae also showed decreased infectivity since the lowest assayed concentration. Our results indicate that a short-term exposure to malathion at levels potentially present in the surface water in environments inhabited by *Chordodes nobilii* affects significantly its preparasitic stages and the infective capacity of parasitic larvae.

Keywords Nematomorpha · *Chordodes nobilii* · Malathion · Bioassays · Infection index

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Introduction

The gordiids (Nematomorpha) spend part of their life cycle in different types of freshwater habitats (i.e.: lakes, streams, rivers, ponds). They go through three developmental stages, a preparasitic free-living larva that penetrates the host and develops into a parasitic juvenile, and a postparasitic free-living adult (Schmidt-Rhaesa 2001, 2002; Hanelt and Janovy 2003; de Villalobos et al. 2003; de Villalobos and Ronderos 2003). A single collection locality such as a stream can contain worms entwined in vegetation at the periphery, between rocks on the bottom, free swimming, wound around submerged sticks, or entangled in a knot near the substrate (Hanelt et al. 2005). The females lay eggs in gelatinous strings that are attached to bottom substrates. At hatching, larvae do not exceed 100 μm in length and bear hooks and stylets on the anterior end, with which they are able to penetrate paratenic or definitive hosts.

Insects, including those of medical and agronomical importance, are regarded as the most frequent definitive hosts, and gordiid infection may lead to severe damage or even death (de Villalobos and Miralles 1997; de Villalobos et al. 1999; Schmidt-Rhaesa and Ehrmann 2001).

Many freshwater environments such as those belonging to the Sauce Grande basin, Buenos Aires, Argentina, are

being polluted due to activities usually undertaken in intensively cultivated nearby areas. The Sauce Grande Basin is a predominantly agricultural and cattle-raising area, with small urban settlements such as Sierra de la Ventana (38°9′0″S, 61°48′0″W), where the current euthrophication level of the water system has been attributed to an increase in the use of fertilizers, to hydric erosion by overgrazing and increased land-use intensity, and to population growth (Vouilloud et al. 2005). Agriculture lands cover 28% of the total area, the main crops being wheat, oat, sunflower, grain sorghum, maize and soy; chemical insecticides are used in 17% of the crop fields (Aduriz et al. 2003). Several pesticides reach surface waters by runoff from fields or direct discharge and represent a potential risk for both horsehair worms and other aquatic organisms.

Malathion (Butanedioic acid, [(dimethoxyphosphinothioyl) thio]-Diethyl ester), is an organophosphate (Op) insecticide classified by the EPA as a toxicity class III pesticide and as a general use pesticide (GUP). It is widely used because of its relatively low toxicity to mammals and high selectivity for insects compared with other organophosphorus insecticides (Pandey et al. 2005). Malathion specifically is toxic to invertebrates because they are not able to metabolize and excrete malathion as quickly as vertebrates. In insects, Ops not only inhibit acetylcholinesterase (AChE) but also alter carbohydrate and protein metabolisms, thus increasing lethality (Cook et al. 2005).

Malathion is used for insect control on agricultural crops, on stored products, on golf courses, in home gardens, and in outdoor sites; it is also used to kill mosquitoes and Mediterranean fruit flies (medflies) in large outdoor areas. Additionally, malathion is used to kill fleas on pets and to treat head lice on humans. It is usually sprayed on crops from an airplane over wide land areas. Malathion can potentially be released to surface waters by direct application, storm runoff from sprayed fields or urban/residential areas, atmospheric deposition following aerial application (wet deposition from rain and fog water), waste water releases from formulation, manufacturing or processing facilities, and spills (ATSDR 2003).

Malathion in water usually undergoes chemical and microbial degradation within a few weeks, but it can remain in the environment for months. The rate and extent of its degradation is dependent on the chemical and physical properties of the water system, particularly temperature and pH, and on the composition of the microbial population present in the system. Malathion is rapidly degraded in aqueous solution at pHs 9 and 7.7, with half-lives of $\sim$12–24 h and >3 days, respectively (ATSDR 2003). Cook et al. (2005) reported that the half-life of malathion in freshwater was 12 days and that it became undetectable 4 weeks after being experimentally released into the aquatic environment.

The toxic effect of malathion on different aquatic organisms has been studied by several authors (e.g.: Cripe 1994; Tsuda et al. 1997; Key et al. 1998; Khangarot and Ray 1988; Pathiratnea and George 1998; Lund et al. 2000; Bonfanti et al. 2004; Pandey et al. 2005; Key and Fulton 2006; Budischak et al. 2008. There is very little information on this subject for nematomorphs, despite the fact that their habitats receive numerous chemicals from agricultural runoff and domestic and industrial wastes.

Therefore, the objective of this study is to evaluate the effect of short-term exposure to malathion concentrations presumably present in the environment on preparasitic stages (eggs and larvae) of *Chordodes nobilii* (Gordiida, Nematomorpha).

Materials and methods

Adults of *C. nobilii* were collected from numerous streams of the Sauce Grande Basin, Buenos Aires, Argentina.

Once in the laboratory, individuals from each stream were kept in separate containers with aerated dechlorinated tap water, at a room temperature of 23 ± 1°C, for 10 days. After mating, females were held individually in containers for oviposition. Bioassays were performed with embryos (mainly in blastula stage) and larvae.

The test substance was a malathion formulation (100% w/v) obtained from SENASA (National Service of Health and Agroalimentary Quality, Ministry of Production, Argentine.

A stock solution of 73 mg a.i. malathion/l was prepared in distilled water. The concentration was checked by the GC-NPD method using a Hewlett–Packard model 6890, (detection limit 0.01 µg/l; sensitivity <0.02 µg/l). This stock solution was diluted to obtain the test solutions.

The dilution medium used in exposure assays was reconstituted hard water (pH 7.99, hardness 162 mg CO_3Ca/l), with the following chemical composition (mg/l): $NaHCO_3$ 192, $CaSO_4.2\ H_2O$ 120, $MgSO_4$ 120, KCl 8, (Weber 1993). The dilution medium was chosen because it provides values of water hardness similar to those at natural collection sites.

The experiments were carried out following protocols previously detailed by Achiorno et al. (2008). The bioassays with embryos and larvae were performed at the following nominal concentrations expressed as µg a.i. malathion/l: 0 (control), 36, 55, 73, 146 and 220. Each concentration including controls was tested in triplicate, in a rearing chamber at 23 ± 1°C, under semi-static conditions.

Also, experiments were conducted in the dark to avoid excessive breakdown of malathion through photodegradation (De Guise et al. 2004).

Embryo bioassays

We selected pieces of egg strings having embryos mainly in blastula stage (May 1919), that had been laid by field-collected females.

The bioassay consisted of the following three consecutive periods:

1. *Exposure*: embryos remained for 96 h in the assay media, which were partially renewed every day and entirely replaced by control medium at the end of the experiment.
2. *Post-exposure*: the containers with embryos previously exposed to the test chemicals were periodically checked until the appearance of free-living larvae (FL), using light microscopy. To determine the amount of FL, ready-to-hatch larvae within egg capsules (LC), non-viable embryos (NE) with collapsed envelopes and patches of amorphous material, a sample of each replicate was taken and 100 individuals were randomly selected.
3. *Infection of host insect* (Exposure of *Aedes aegyptii* (Diptera) larvae to *C. nobilii* larvae): On the basis that *C. nobilii* larvae have scarce activity, viability was determined by evaluation of their infective capacity with the Infection Index Mean Abundance, IIMA, as total number of *C. nobilii* larvae that could infect *A. aegypti* larvae divided by total number of *A. aegypti* larvae observed (Bush et al. 1997) under light microscopy. This period was started when the proportion of FL in each sample from the post-exposure period (period 2) was nearly 50%. *A. aegyptii* (Diptera) larvae were exposed to *C. nobilii* larvae for 72 h under control conditions.

The embryo bioassay was considered valid when the proportion of NE in the control groups was $\leq$10%.

In embryo bioassay the endpoints were number of NE and number of viable embryos (V), which includes FL and LC, at the end of the second period, and the IIMA at the end of the third period.

Larva bioassay

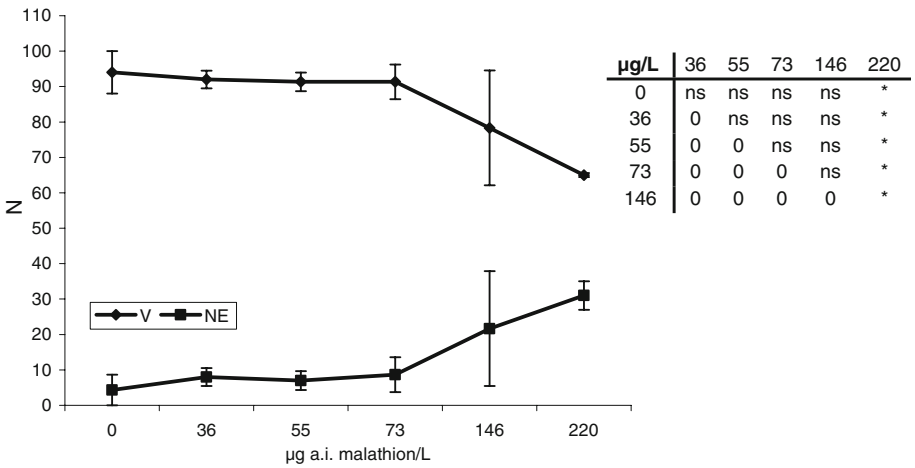
Pieces of egg strings were rinsed in a 1:250 Clorox bleach solution (Hanelt and Janovy 1999), and placed in 50-ml plastic containers until the FL stage was reached. The experimental protocol consisted of two consecutive periods: in the first one, *C. nobilii* FL were exposed to malathion for 48 h, with partial renewal of the solution every 24 h. In the second period, *A. aegyptii* larvae were exposed to *C. nobilii* FL for 72 h in control medium.

The IIMA, used as a parameter of the infective capacity of *C. nobilii* to the host (*A. aegyptii*), was chosen as endpoint. This bioassay was considered valid when IIMA in the control group was $\geq$2.

Significant differences between the control and treated groups were determined using one-way ANOVA followed by Tukey's post-hoc comparison test. The Shapiro–Wilk test and the Levene median test were used to assess normality and homogeneity of variance of data, respectively. An arcsin transformation was applied to the IIMA (Zar 1999). The significance level was set at $P < 0.05$. Statistical analyses were performed using Infostat software.

Results

In the embryo bioassay, the number of NE was significantly different from the control only for the highest concentration (220 µg malathion/l). However, it is noteworthy that there was a slight increase in the number of NE with increasing test concentrations despite the lack of statistically significant differences from the control (Fig. 1). Alterations in body shape were observed for both FL and LC exposed to malathion, as compared to those in the control groups (Fig. 2b). Although no inhibition of



µg/L	36	55	73	146	220
0	ns	ns	ns	ns	*
36	0	ns	ns	ns	*
55	0	0	ns	ns	*
73	0	0	0	ns	*
146	0	0	0	0	*

Fig. 1 Embryos viables (V) and no viables (NE). Value expressed as arithmetic mean by treatment ± SD. The text box of Tukey's comparisons is shown. * Differ statistically significant ($P < 0.05$), ns: not significant

embryonic development was detected at the concentrations assayed, there was a remarkable decrease in the infective capacity of larvae hatched from exposed eggs, consistent with a concentration-dependent effect from the lowest assayed concentration (36 a.i. µg/l); the IIMA showed significant differences between the treated and the control groups (Fig. 3).

A decrease in the infective capacity (IIMA) with increasing malathion concentrations was also observed in the directly exposed larvae from the lowest assayed concentration (36 a.i. µg/l), showing significant differences with respect to the control and a concentration–dependent effect (Fig. 4).

Discussion

The toxic effects of xenobiotics on Gordiida have begun to be investigated recently. Until now, the sensitivity to glyphosate was only evaluated for a Gordiida species, *Chordodes nobilii* (Achiorno et al. 2008). Thus, the present work represents the first report of the effect of an insecticide on a species of the Phylum Nematomorpha.

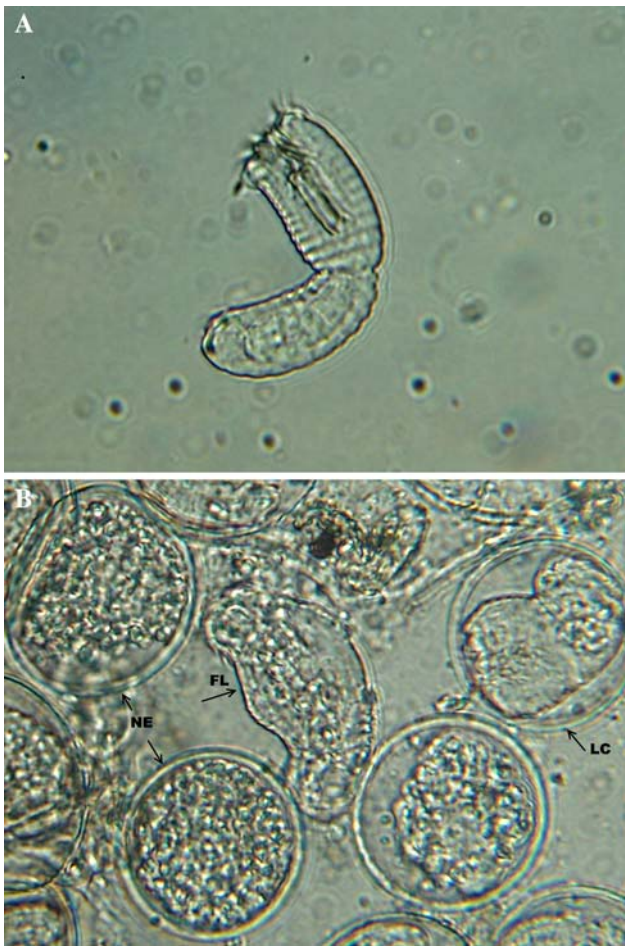


Fig. 2 Light microscopy view (X 400) of free-living larvae (FL). **a** Normal FL. **b** abnormal FL, abnormal ready-to-hatch larvae within egg capsules (LC) and non-viable embryos (NE)

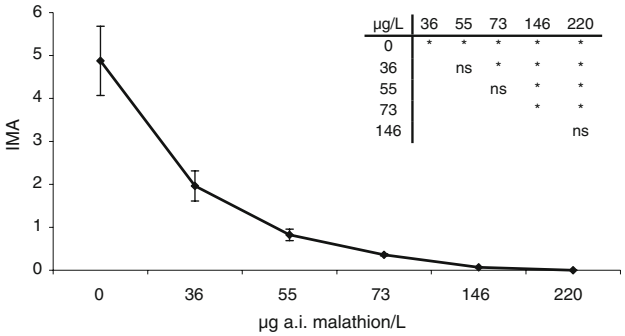


Fig. 3 Infection index mean abundance (IIMA) observed for the control and the assay concentrations in the embryonic development's assays. Value expressed as arithmetic mean ± SD by treatment. The text box of Tukey's comparisons is shown. * Differ statistically significant ($P < 0.05$), ns: not significant

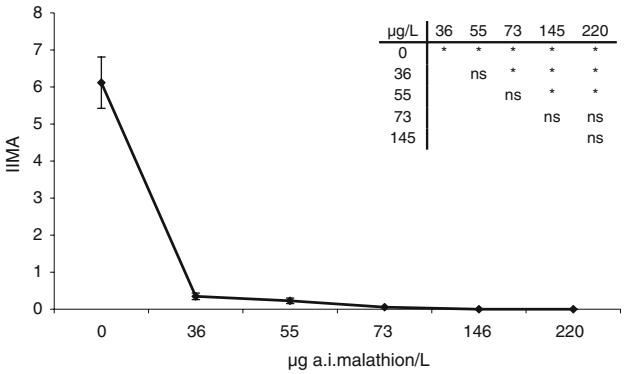


Fig. 4 Infection index mean abundance (IIMA) observed for the control and the assay concentrations in larvae's assay. Value expressed as arithmetic mean ± SD by treatment. The text box of Tukey′s comparisons is shown. * Differ statistically significant ($P < 0.05$), ns: not significant

Some of the LC50 96-h values (µg/l) of malathion reported for different test organisms are as follows: 0.76 for the amphipod *Gammarus fasciatus* (Johnson and Finley 1980); between 5.4 and 83 for juveniles and postlarvae of many species of estuarial crustaceans (Cripe 1994); 7.2, 20.5 and 24.3 for the nauplii, copepodites and ovigerous females, respectively, of the marine copepod *Tigriopus brevicornis* (Forget et al. 1998); and between 2.29 and 1.26 for other amphipods (Leight and Van Dolah 1999). Finally, Key and Fulton (2006) reported LC50 values (µg/l) of 8.94, 13.26 and 39.92 and 96-LOEC values (µg/l) of 3.75, 12.5 and 25.00 for recently hatched larvae, 18-day larvae and postlarvae, respectively, of *Palaemonetes pugio*. The sensitivity of the developmental stages of *C. nobilii* to malathion obtained in the present study is lower than that previously reported for some crustaceans.

The black fly larvae of *Simulium vittatum* (Diptera: Simuliidae), commonly found on rocks and other substrates in streams and rivers, were used by Overmyer et al. (2003) to test the effect of malathion. These authors obtained a 96 h CL 50 of 54.20 μg/l, and taking into account that this species and *C. nobilii* live in similar habitats, we might expect a higher sensitivity for the latter.

Among vertebrates, Bonfanti et al. (2004) reported no inhibition of development in *Xenopus laevis* blastulae at concentrations between 0.375 and 6 mg/l, but malformations were found. In assays using larvae of *Rana boylii*, Sparling and Fellers (2007) found a 96 h-LC50 of 2.14 mg/l. In fish, the hatching rate of zebrafish eggs was near 100% for malathion concentrations between 0.25, and 2 mg/l, with tail malformations in pro-larvae at all tested concentrations (Fraysse et al. 2006). Cook et al. (2005) exposed zebrafish embryos to malathion concentrations between 0.5 and 3 mg/l, finding malformations at concentrations equal to or greater than 2.5 mg/l, and a reduced hatching percentage at concentrations higher than 2.0 mg/l as compared with the controls. So, zebrafish embryos were more sensitive to malathion after hatching than younger embryos. Similar results were obtained in the present study, with *C. nobilii* embryos being less sensitive than larvae to malathion.

Budischak et al. (2008), exposed embryos of the pickerel frog, *Rana palustris*, to three environmentally realistic concentrations of malathion (15, 60 and 600 μg/l), which are of the same order as those used in the present paper. The authors found that malformation frequency increased and both hatching and viability decreased as malathion concentrations increased. They also observed that tadpoles exposed to malathion concentrations equal and above 60 μg/l during embryonic development showed increased susceptibility to trematode infection (as reflected by a significant increase in the frequency of cysts).

Our results and those mentioned above suggest that malathion affects host-parasite interactions. *Rana palustris* tadpoles exposed to malathion during embryonic development showed an increase in susceptibility to parasites with increasing toxicant concentrations, while *C. nobilii* larvae exposed to malathion during embryonic development showed a decrease in their infective capacity with increasing toxicant concentrations. On this basis, the populations of terrestrial insect species (e.g. mantids), which are the main definitive hosts of Gordiida (Schmidt-Rhaesa and Ehrmann 2001), would increase in areas where malathion is used.

Taking into account the results reported above for other animals, *C. nobilii* could be included among the species sensitive to malathion: a brief exposure (in the order of 10% of the embryonic phase) during early embryonic period to different malathion concentrations, caused an inhibition of the development at 220 a.i. μg/l and a decline infective capacity of larvae hatched from exposed eggs, from the lowest concentration tested (36 a.i. μg/l). Directly exposed larvae exhibited a sharp decrease in their infective capacity of more than 50% from 36 a.i. μg/l as compared to the control. In addition, the embryonic stage of *C. nobilii* is likely to be less sensitive than the larval one, despite the occurrence of alterations in the body shape of larvae developed from exposed eggs.

According to the pesticide registration rejection rate analysis (USEPA 1994) our results indicate that malathion is highly toxic for embryos and larvae of *C. nobilii*. Based on the considerations mentioned above and taking into account that the doses indicated for general agriculture are between 0.175 and 6.25 lbs malathion ai/Acre, the estimated environmental concentration in water possibly ranges between 291 and 2.94 μg malathion/l (USEPA 2006). These malathion concentrations possibly found in the environment would pose a toxic risk to *C. nobilii* embryos and larvae.

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