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Research Article

Assessment of the effect of a commercial formulation of Methomyl on reproduction of *Daphnia obtusa* Kürz (1874)

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ABSTRACT. The aim of this study was to evaluate the effect of a commercial formulation of Methomyl on the reproduction in *Daphnia obtusa*. Neonates (<24 h) were exposed to concentrations of insecticide (1.0, 1.5, 2.0, 2.5, 3.0 µg L^{-1}) for a period of 21 days. The results showed a significant decrease in the number of molts, total neonates, fertility and sex ratio index. Significant regressions were found between reproductive parameters and concentrations of the insecticide: molts (R^2 = 0.96), fertility (R^2 = 0.97), number of female neonates born per female (R^2 = 0.90), and the number of male neonates born per female (R^2 = 0.94). Embryotoxicity was also observed; the number of neonates born with malformations increased in the highest concentrations of Methomyl 90 SP® tested. In conclusion, Methomyl 90 SP® inhibits reproduction of *D. obtusa*. However, a clear effect as an endocrine disrupter was not observed. This insecticide is embryotoxic; it causes malformations in neonates of *Daphnia obtusa*.

Keyswords: *Daphnia*, endocrine, neonates, pesticide, disruptor, zooplankton, ecotoxicology.

Evaluación del efecto de una formulación comercial de Metomil sobre la reproducción de *Daphnia obtusa* Kürz (1874)

RESUMEN. El objetivo de este trabajo fue evaluar el efecto de una formulación comercial de Metomil sobre la reproducción en *Daphnia obtusa*. Para ello, neonatos de *D. obtusa* (<24 h) fueron expuestos a concentraciones del insecticida (1.0, 1.5, 2.0, 2.5, 3.0 µg L^{-1}) por un período de 21 días. Los resultados mostraron una disminución significativa en el número de mudas, recién nacidos totales, fertilidad e índice de proporción de sexos. Se encontraron regresiones significativas entre algunos parámetros reproductivos y las concentraciones del insecticida: mudas (R^2 = 0.96), fertilidad (R^2 = 0.97), número de neonatos hembras por hembra (R^2 = 0.90) y el número de recién nacidos machos (R^2 = 0.94). También se observó embriotoxicidad, el número de neonatos nacidos con malformaciones aumentó en las concentraciones más altas de Metomil 90 SP® probadas. En conclusión, el Metomil inhibe la reproducción de *D. obtusa*, aunque no se observó en forma clara un efecto como disruptor endocrino. Este insecticida es embriotóxico, ya que provoca malformaciones en los neonatos de *Daphnia obtusa*.

Palabras clave: *Daphnia*, neonatos, pesticidas, disruptor endocrino, zooplancton, ecotoxicología.

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INTRODUCTION

Pesticides are usually non target-specific, and therefore may cause harm to non-target species; many are quite persistent for long periods in the environment (Palma *et al*., 2009). Pesticides are characterized by their high toxicity to aquatic organisms (Pereira & Goncalves, 2007). Currently, there is growing interest in determining the effects that endocrine disruptors may have on these compounds in aquatic organisms, which can enter aquatic ecosystems from crop sprayings runoff in agricultural lands (Mazurová *et al*., 2008; Palma *et al*., 2009; Yang *et al*., 2010).

Methomyl, *S*-methyl (*EZ*)-*N*-(methylcarbamoyloxy) thioacetimidate is an oxime carbamate insecticide. It was introduced in 1966 as a broad spectrum insecticide. It is also used as an acaricide to control ticks and spiders. It is used for foliar treatment of vegetables, fruits and field crops, as well as cotton and commercial ornamentals. It is also used as fly bait. Methomyl is effective in two ways: (a) as a contact insecticide because it kills target insects upon direct contact, and (b) as a systemic insecticide because of its capacity to cause overall systemic poisoning in target insects, after it is absorbed by and transported through the pests that feed on treated plants (Wasim *et al*., 2010). In Chile the use of Methomyl is authorized by the Servicio Agricola y Ganadero (SAG, 2013).

Methomyl varies from moderate to highly toxic in certain non-target aquatic organisms. The cladoceran *Daphnia magna* is very sensitive to the chemical with a $CL_{50\text{-}48h}$ of 32 mg L^{-1} and a chronic maximum acceptable concentration in the range of 1.6 to 3.5 mg L^{-1} for other species (WHO, 2004; Pereira & Goncalves, 2007). Mano *et al*. (2010) reported that sensitivities to Methomyl were highly correlated among cladoceran species, but the co-tolerance level varied markedly among species. *Ceriodaphnia reticulate* showed the highest sensitivity, whereas *Moina macrocopa* and *Scapholeberis kingi* showed the lowest sensitivity. These authors indicated that the degree of chemical impacts on natural communities can vary depending on the cladoceran species composition. Methomyl has been described as an estrogenic endocrine disruptor which weakly stimulates aromatase activity (Andersen *et al*., 2002; Cocco, 2002; Mckinlay *et al.*, 2008).

Cladocerans, known as water fleas, are small crustaceans inhabiting lakes and ponds as zooplankton. They are major constituents of food webs, acting as grazers, consuming phytoplankton, and as prey items for invertebrate and vertebrate predators (Oda *et al*., 2005). Most daphnids are cyclic parthenogenetic species that exhibit both asexual and sexual reproduction (Dodson & Frey, 1991). Certain environmental conditions such as reduced photoperiod, low food levels and high density have been shown in laboratory settings to trigger male offspring production (Hobaek & Larsson, 1990; Kleiven *et al*., 1992). Perturbations in sexual development in daphnids have also been used as an endpoint following exposure to endocrine-disrupting chemicals. The development of sexual characteristics in daphnids apparently involves hormonal processes during juvenile development and maturation (Mitchell, 2001).

In response to some environmental factors, daphnids start sexual reproduction; males appear and females start producing mictic eggs, which will be fertilized by the males. The fertilized eggs are in a state of diapause (resting eggs), and can survive in severe environments. Environmental factors such as short day length, food depletion and high population density are known to be keys to the initiation of sexual reproduction (Zhang & Baer, 2000; Tatarazako & Oda, 2006; Baer *et al.*, 2009). Crustaceans are organisms that may be targets of pesticides in freshwater ecosystems and disturb their endocrine system. The aim of this study was to evaluate the effect of Methomyl on reproductive traits in *Daphnia obtusa.*

MATERIALS AND METHODS

The *Daphnia obtusa* test organisms were obtained from the Laboratory of Ecotoxicology, Faculty of Science, University of Valparaiso. The environmental conditions were according to NCh.2083 (1999): controlled photoperiod of 16 h light and 8 h dark, 20 ± 2°C, hardness 120 ± 25 mg L^{-1} (as $CaCO_3$), dissolved oxygen above 80% saturation and pH 7.0 ± 2. The organisms were fed daily by providing 2.5×10^5 cells mL^{-1} $daphnia^{-1}$ of the green microalga *Chlorella* sp. (Baer *et al.*, 2009).

The pesticide used was the commercial formulation of Methomyl 90 SP® Hydro DowAgroScience (www.dowagro.com/cl), whose composition is 90% active ingredient and 10% inert ingredients. This formulation is a powder with solubility in water of 57.9 g L^{-1}, pH 6.91 ± 0.07 and a stability of 30 days. The stock solution was prepared with distilled water in a 500 mL volumetric flask glass. The solution was kept at 4°C in darkness and renewed at 14 days. Test solutions were prepared using this solution.

Experimental design

Neonates (<24 h old) were exposed to five increasing concentrations of commercial Methomyl 90 SP® and control (non-toxic). The test concentrations were: 1.0, 1.5, 2.0, 2.5 and 3.0 µg L^{-1} in 40 mL glass vessels. Each treatment consisted of ten replicates and one female was placed in each replica. The exposure time was 21 days. To provoke the stimulation of production of males, females were exposed to altered photoperiod conditions and the amount of food reduced (Zhang & Baer, 2000, Baer *et al.*, 2009). During the bioassay the photoperiod was 8 h light/16 h dark. The daphnids were fed daily with 50000 cells mL^{-1} of *Chlorella* sp. (Baer *et al*., 2009). In each culture medium of daphnids the concentrations were renewed three times per week. The environmental temperature was 20 ± 0.2ºC. The response variables measured were: number of males born per female, number of molts per female and number of malformed neonates per female according to Leblanc *et al*. (2000); under developed first antennae, curved shell spine, unextended shell spine and survival at 21 days of exposure. Observations were made with a stereomicroscope. Culture conditions such as pH, conductivity, dissolved oxygen and temperature were measured weekly.

All data was first tested for normality and homogeneity of variance to meet statistical demands. The one-way analysis of variance followed by Tukey multiple comparison tests was used to determine which concentration produced responses that were different from the control. The level of statistical significances applied was $P < 0.05$. In addition, regressions were performed between biological responses and concentrations of Methomyl. The SYSTAT 5.0 Statistics was used.

RESULTS

Figure 1 show that the number of molts decreased significantly in comparison to control with average of 9 ± 0.7 molts. The regression with the concentration of insecticide was significant (Table 1). On the other hand, the number of born females decreased significantly at all Methomyl 90 SP® concentrations (Fig. 2). We found a significant regression between the number of females born per female and concentrations of the insecticide (Table 1). A significant decrease in the total number of neonates per female with increasing concentrations of Methomyl 90 SP® was observed (Fig. 3). The number of males born per female decreased significantly from 1.0 µg L^{-1} (Fig. 4). Regarding the sex ratio index, it was higher in the control and lower in concentration of 3.0 µg L^{-1} (Fig. 5). The number of neonates with malformations increased with increasing concentrations of Methomyl 90 SP® (Figs. 6, 7; Table 1).

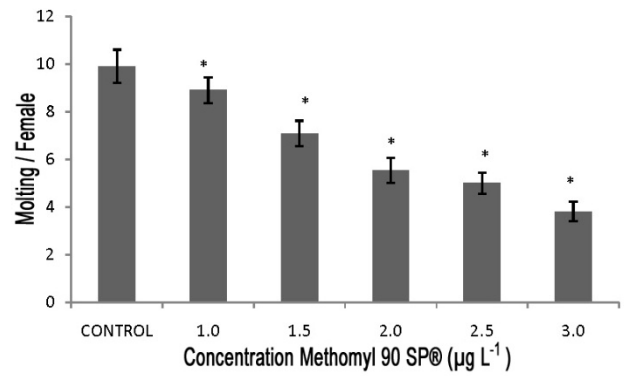

Figure 1. Mean number of molts per female exposed to different concentrations of Methomyl 90 SP®. *Indicates a significant difference from the control group ($P < 0.05$).

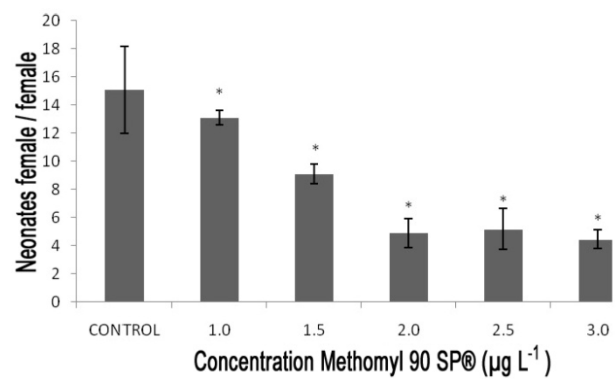

Figure 2. Mean of female neonates per female exposed to different concentrations Methomyl 90 SP®. *Indicates a significant difference from the control group ($P < 0.05$).

DISCUSSION

In this study, Methomyl caused a decrease in the molting process of *D. obtusa*. This decrease could be explained by the alteration of the X-organ, affecting the secretion of molting inhibitory hormone (MIH), and inhibiting the production of ecdysteroids by the Y-organ (Mazurová *et al*., 2008). This altered the duration of the molting cycle and the start of reproduction. The anti-ecdysteroid effect was also observed with atrazine and endosulfan (Palma *et al.*, 2009). However, our results differ from those reported by Pereira *et al.* (2010), who found that Methomyl affected molting-related gene transcription. Methomyl strongly up-regulated molting-related genes, including those coding for various structural constituents of the cuticle, cuticular proteins and chitin deacetylases; which suggests that the molting cycle was accelerated in response to the chemical exposure.

According to the hypothesis, an increasing concentration of pesticide should cause an increase in the number of female hatchlings produced. However, the born females decreased. These results could be due to the toxicity of pesticide that inhibits the endocrine effect. On the other hand, survival and growth depend on energy availability, and pesticides are known to reduce cellular energy budgets in daphnids (De Coen & Janssen, 2003; Barata *et al*., 2004). Pereira *et al.* (2010) reported that Methomyl promoted differential transcription of energy-related genes in *Daphnia magna*. They found that Methomyl caused the induction of mRNAs of genes coding for ATP synthase and enzymes involved in glycolysis, and in the respiratory chain, suggesting that the organism needs energy to cope with the environmental challenge.

Table 1. Simple regressions between reproductive traits and Methomyl 90 SP ® concentrations. * Significant regression ($P < 0.05$).

Reproductive traits	Equation	R^2	*P*
Molts	y = -2,.487x + 10.293	0.96*	0.000
Female neonates	y = -4.0291x + 15.349	0.90*	0.004
Total neonates	y = -2.894x + 25.521	0.94*	0.001
Male neonates	y = -2.6777x + 8.2649	0.94*	0.001
Malformed neonates	y = 1.6202x + 0.2523	0.93*	0.002
Sex ratio index	y = -0.1008x + 0.3886	0.39	0.180

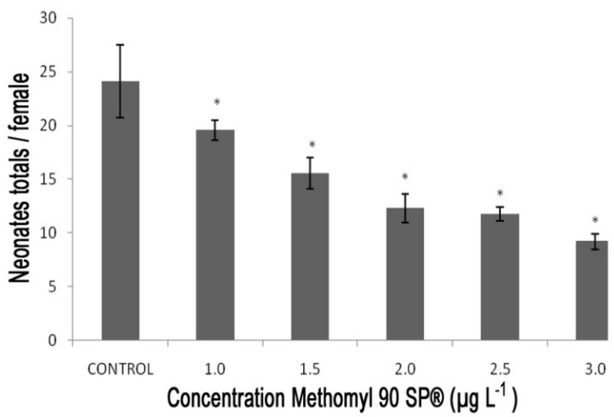

Figure 3. Mean of total neonates per female exposed to different concentrations of Methomyl 90 SP®. *Indicates a significant difference from the control group ($P < 0.05$).

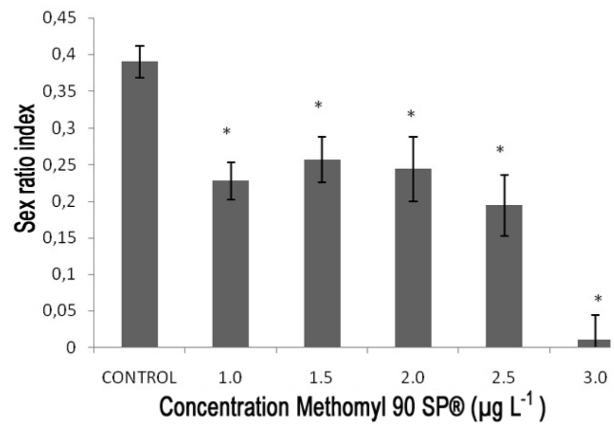

Figure 5. Mean of the sex index at different concentrations of Methomyl 90 SP®. *Indicates a significant difference from the control group ($P < 0.05$).

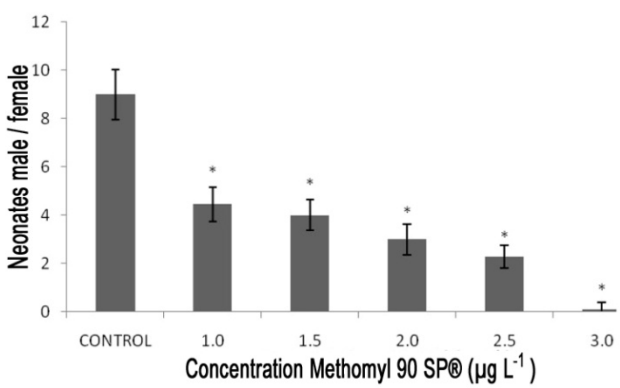

Figure 4. Mean of male neonates per female exposed to different concentrations of Methomyl 90 SP®. *Indicates a significant difference from the control group ($P < 0.05$).

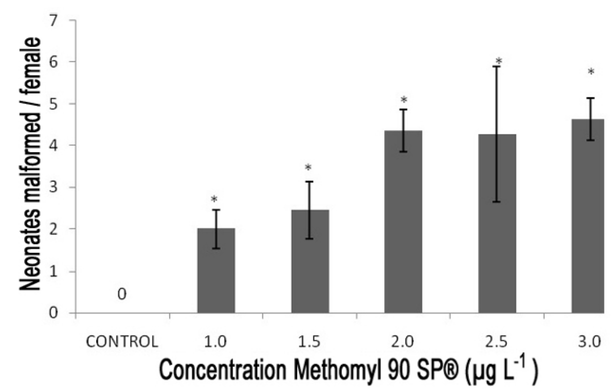

Figure 6. Mean of malformed neonates per female exposed to different concentrations of Methomyl 90 SP®. *Indicates a significant difference from the control group ($P < 0.05$).

The stress conditions in this study (decrease of food and reduced photoperiod) suggest an increase in the toxicity of Methomyl 90 SP® even at lower concentrations. Pereira & Goncalves (2007) showed that zooplankton responds to natural stress conditions, predation pressure and changes in food availability. Similar results were obtained by Zhang & Baer (2000), who found that a decrease in food availability and altered photoperiod increased the toxic effect of a solvent in *D. magna*. In addition, the decline in

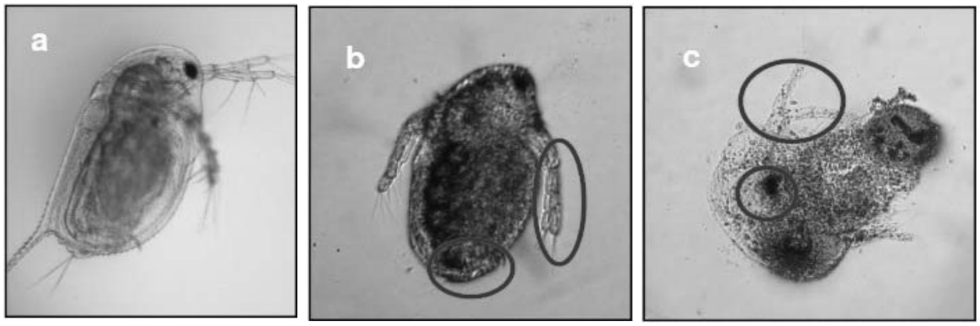

Figure 7. a) Neonate in normal conditions, b) malformed neonate showing curved apical spine and antennae, c) developed neonate, only the compound eye can be observed, no evidence of development of digestive system.

fertility (total neonates produced per female), at different concentrations, could be attributed to the purity of the Methomyl solution, since according to Pereira & Goncalves (2007) some auxiliary chemical pesticides are toxic to *Daphnia* species; in the present study the commercial formulation was used, which could have other unknown “inert” ingredients. This information is not available on the commercial label product. Surfactants and other so-called inert ingredients which are added to the active ingredient to enhance its chemical and physical efficacy as a pesticide, may also contribute significantly for its overall toxicity (Pereira *et al*., 2009).

The sex ratio index was higher in the control. This could be related to the report by Mazurová *et al.* (2008), in that the activity of androgenic gland hormone (GHA), responsible for the generation of males in the endocrine system in crustaceans, can be affected by the toxic effect of a pesticide and act as an endocrine disruptor. The fertility and development of daphnids is directly related to the mandibular organ, responsible for the secretion of the hormone methyl farsenoate (MT), which relates to the generation of male neonates in the molting process (Palma *et al*., 2009). Therefore, the results suggest that the insecticide Methomyl causes hormone inhibition of MT and/or hormone receptors, thus acting as an endocrine disruptor (Rodríguez *et al*., 2007).

The malformations indicate embryotoxicity, generating abnormal development of neonates, partial arrest of early embryonic development, abnormalities in the apical spine and the development of the antenna. Similar results were reported by Kast-Hutcheson *et al*. (2001), who found that the exposure of *Daphnia magna* to propiconazole interferes with the later stages of daphnid embryonic development, and that the toxicity was manifested largely via maternal exposure to the fungicide. Leblanc *et al*. (2000) reported that 4-nonylphenol, which is the alkylphenol degradation product in *D. magna*, caused an increased level of testosterone, which in turn generated malformations in neonates. On the other hand, Palma *et al.* (2009) reported that endosulfan sulphate, that is the transformation product of endosulfan, increased embryo deformities in all concentrations tested on *D. magna*.

In conclusion the exposure of *D. obtusa* to the commercial formulation Methomyl 90 SP® inhibits reproduction of *D. obtusa*. However, stress conditions (photoperiod alteration and low level of food) were not sufficient to determinate the endocrine effect, as these conditions increased the susceptibility of *D. obtusa* to the toxicity of Methomyl 90 SP®. The commercial formulation of Methomyl used is embryotoxic; it causes alterations in the development of structures in neonates in early development stages.

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