



Interactive effects of a bacterial parasite and the insecticide carbaryl to life-history and physiology of two *Daphnia magna* clones differing in carbaryl sensitivity

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ABSTRACT

Natural and chemical stressors occur simultaneously in the aquatic environment. Their combined effects on biota are usually difficult to predict from their individual effects due to interactions between the different stressors. Several recent studies have suggested that synergistic effects of multiple stressors on organisms may be more common at high compared to low overall levels of stress. In this study, we used a three-way full factorial design to investigate whether interactive effects between a natural stressor, the bacterial parasite *Pasteuria ramosa*, and a chemical stressor, the insecticide carbaryl, were different between two genetically distinct clones of *Daphnia magna* that strongly differ in their sensitivity to carbaryl. Interactive effects on various life-history and physiological endpoints were assessed as significant deviations from the reference Independent Action (IA) model, which was implemented by testing the significance of the two-way carbaryl × parasite interaction term in two-way ANOVA's on log-transformed observational data for each clone separately. Interactive effects (and thus significant deviations from IA) were detected in both the carbaryl-sensitive clone (on survival, early reproduction and growth) and in the non-sensitive clone (on growth, electron transport activity and prophenoloxidase activity). No interactions were found for maturation rate, filtration rate, and energy reserve fractions (carbohydrate, protein, lipid). Furthermore, only antagonistic interactions were detected in the non-sensitive clone, while only synergistic interactions were observed in the carbaryl sensitive clone. Our data clearly show that there are genetically determined differences in the interactive effects following combined exposure to carbaryl and *Pasteuria* in *D. magna*.

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1. Introduction

The study of combined effects of multiple chemical stressors is becoming increasingly important in ecotoxicology. This is because the toxicity of a given mixture of chemical stressors can usually not be predicted in a straightforward way from the toxicity of the different individual stressors in that mixture due to non-additive (i.e., interactive) effects. This considerably complicates environmental risk assessment of chemical mixtures (van Gestel et al., 2010). In addition, chemical stressors can also interact with (biotic and abiotic) 'natural' stressors. It is well-documented that 'natural' stressors such as temperature and food limitation may modify the effects of chemicals on organisms and vice versa (see

recent reviews of Heugens et al., 2001; Holmstrup et al., 2010; Laskowski et al., 2010). A meta-analysis of interactions between natural stressors and toxic chemicals in 61 studies by Laskowski et al. (2010) showed a significant interaction in 62.3% cases, indicating the importance of the occurrence of such interactions in natural ecosystems. Moreover, these authors showed that the null hypothesis assuming no interactions between chemical and natural stressors should be rejected at $p = 2.7 \times 10^{-82}$. The review by Holmstrup et al. (2010) evaluating the interactive effects of binary combinations of natural and chemical stressors as reported in more than 150 studies (covering natural stressors including heat, cold, desiccation, oxygen depletion, pathogens and immunomodulatory factors) revealed similar results. In this set of studies, synergistic interactions, i.e. with the effect of the combination of two stressors being stronger than expected based on their non-interactive combined action, were reported in more than 50% of the cases. These authors also report antagonistic interactions, i.e. where the effect of combined stressors is smaller than expected, but these interactions

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were found in many fewer cases. Holmstrup et al. (2010) also pointed out that synergistic effects of chemical and natural stressors appear to be more likely with increasing levels of stress caused by one or both stressors. The aim of the present study was to start testing this hypothesis from a slightly different angle by investigating whether a clone of the water flea *Daphnia magna* that is more sensitive to a given chemical, and thus experiences a higher level of stress, would also experience more pronounced synergistic effects during a combined exposure to a natural stressor and that chemical compared to a less sensitive clone.

We chose the insecticide carbaryl and the bacterium *Pasteuria ramosa*, a bacterial endoparasite of *D. magna*, as our model system for a combined analysis of a chemical and natural stressor. Earlier work found synergistic effects for these two stressors in *D. magna*. Coors et al. (2008) and Coors and De Meester (2008, 2011) exposed a single clone of *D. magna* to the insecticide carbaryl and *P. ramosa* and found that sublethal concentrations of carbaryl enhanced the virulence of the parasite: i.e. sterilization of *D. magna* by *P. ramosa* was accelerated under carbaryl exposure. In addition, Jansen et al. (2011a) showed in an experimental evolution trial that the evolution of increased resistance to the pesticide carbaryl resulted in an increased susceptibility to infection by *P. ramosa*.

We performed a 10-day exposure experiment according to a full-factorial $2 \times 2 \times 2$ design, using two *D. magna* clones (one clone sensitive to carbaryl, denoted 'S', and one clone non-sensitive to carbaryl, denoted 'NS'), *P. ramosa* (absence vs. presence) and carbaryl (absence vs. presence) as factors. During this experiment we recorded several life-history endpoints (survival, growth and early reproduction). In addition, we included several physiological endpoints such as filtration rate, energy reserves, electron transport system activity, acetylcholinesterase- and phenoloxidase activity. Including these endpoints does not only broaden the set of endpoints but also may help in pinpointing mechanistic causes of interaction effects. Three-way and two-way ANOVA on log-transformed observational data were then used to test for interactive effects between carbaryl and *P. ramosa* on all recorded *D. magna* endpoints and to test whether interactions differed between the sensitive (S) and non-sensitive (NS) clone.

2. Materials and methods

2.1. Organisms and stressors

Daphnia magna is a planktonic cyclic parthenogenetic crustacean and a keystone species in freshwater lakes and ponds (Lampert, 2011; Stollewerk, 2010). It is a frequently-used model organism in ecotoxicology (Altshuler et al., 2011) and for host-parasite studies (e.g. Ebert et al., 2004; Decaestecker et al., 2007; Coors et al., 2008).

The gram-positive bacterium *Pasteuria ramosa* is an obligate endoparasite of *D. magna* that irreversibly sterilizes its host within 5 to 15 days after infection (Ebert, 2005). The energetic resources that become available through suppression of reproduction are channeled towards the production of new parasite endospores, which can infect new hosts through horizontal transmission from decaying hosts (Ebert et al., 2004). Susceptibility to *P. ramosa* may depend on genetically and environmentally determined host immunity (Little and Ebert, 2000).

The methyl carbamate insecticide carbaryl is a model substance that is representative for insecticides with mode of action class 1a, i.e. carbamate acetylcholinesterase inhibitors, according to the Insecticide Resistance Action Committee (<http://www.irac-online.org/eClassification>). Carbaryl acts as a quasi-irreversible inhibitor of acetylcholinesterase, an enzyme which hydrolyses the neurotransmitter acetylcholine. Inhibition of acetylcholinesterase results in the accumulation of acetylcholine at the

postsynaptic receptor, which results both in repetitive firing and blocking of other neuronal transmissions (Corbett et al., 1984).

2.2. Experimental design

Two different *D. magna* clones with a known difference in their sensitivity to carbaryl (based on earlier experiments, Jansen et al., 2011a), and further denoted as clone S (sensitive) and NS (non-sensitive), were cultured parthenogenetically under controlled laboratory conditions ($20 \pm 1 \text{ }^\circ\text{C}$, 16:8 h light:dark cycle; 1000 lux) for multiple generations prior to the experiment. The chemically defined ADaM medium (Klüttgen et al., 1994) was used as both the culture and the test medium. Stock cultures as well as experimental animals were fed daily with 2×10^5 cells per mL of the green alga *Pseudokirchneriella subcapitata*, corresponding with 1.25 mgC L^{-1} . Both clones originated from the dormant egg bank of a pond in Oud-Heverlee Zuid, Belgium ($50^\circ 50' 22'' \text{N}$, $4^\circ 39' 18'' \text{E}$), also described by Coors et al. (2009). A three-way full factorial experiment was conducted with parasite challenge (absent or present), carbaryl exposure (absent or present) and clone (S or NS) as factors, resulting in four exposure treatments per clone. Three independent replicates of 320 animals per treatment were set-up in 10 L glass aquaria holding ADaM medium using pooled second to fourth brood juveniles (<24 h old). The population density of the daphnids was maintained at one individual per 5 mL medium during the first four days of the exposure and then changed to one daphnid per 30 mL until the end of the experiment (day 10) by adapting the volume in the aquaria. The densities used during the exposures are realistic for the field, where densities of >300 individuals/L can be observed. The exposures took place under diffuse light conditions (40 cd, 16:8 h light:dark cycle) and under controlled temperature conditions ($20 \pm 1 \text{ }^\circ\text{C}$). The medium was renewed every other day. Temperature (mean \pm SD: $19.3 \pm 0.4 \text{ }^\circ\text{C}$), oxygen concentration (mean \pm SD: $9.05 \pm 0.27 \text{ mg L}^{-1}$), pH (mean \pm SD: 7.63 ± 0.10) and conductivity (mean \pm SD: $892 \pm 20 \text{ } \mu\text{S cm}^{-1}$) did not differ systematically among treatments or replicates. The experiment was terminated after 10 days of exposure. At this point in time, most animals had released their first brood in the control treatment, allowing a reliable assessment of effects on early reproduction.

2.3. Stressor exposures

2.3.1. Parasite challenge

D. magna neonates from an isoclonal stock culture of clone K6 (originating from a pond in Kiel, Antwerp, Belgium and cultured in our laboratory in Ghent for over 20 years) were exposed to sediment from a pond in Knokke, Belgium (Knokke In, $51^\circ 20' 6'' \text{N}$, $3^\circ 20' 54'' \text{E}$), which is known to contain *P. ramosa* spores (Jansen et al., 2010). After 22 days the infected hosts were collected and ground. The resulting suspension was filtered over a $60 \text{ } \mu\text{m}$ nylon filter (Millipore) and then diluted with deionized water to a concentration of 5×10^6 spores mL^{-1} . A placebo-suspension for parasite-free treatments was prepared in the same way by grinding the same amount of uninfected stock culture daphnids, in such a way that it contained an equal weight of ground daphnia tissue per mL. Prior to challenging the daphnids with the bacterial spores, the suspension was examined under a phase-contrast microscope at a $400\times$ magnification to determine the presence of spores from other parasites that may have been present in the sediment. Only *P. ramosa* spores were observed. Daphnids were challenged with 3.75×10^4 mature *P. ramosa* spores per mL medium during the first six days of the experiment. More specifically, spores were added to fresh medium at the start of the experiment (day 0) and at the time of media renewals, i.e. on day two and day four (Jansen et al., 2011b). All parasite-free treatments received the same amount of

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