# Combined exposure to parasite and pesticide causes increased mortality in the water flea Daphnia

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# Combined exposure to parasite and pesticide causes increased mortality in the water flea *Daphnia*

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**Abstract** Organisms are exposed to multiple biotic and abiotic environmental stressors, which can influence the dynamics of individual populations and communities. Populations may also genetically adapt to both natural (e.g. disease) and anthropogenic (e.g. chemical pollution) stress. In the present study, we studied fitness consequences of exposure to both a parasite (i.e. biotic) and a pesticide (i.e. abiotic) for the water flea *Daphnia*. In addition, we investigated whether these fitness consequences change through time as a population evolves. Thus, we exposed *Daphnia magna* clones, hatched from dormant eggs isolated from different time layers of a natural dormant egg bank, to the parasite *Pasteuria ramosa* and the insecticide diazinon in a multifactorial experiment. While our experimental treatments for unknown reasons failed to induce disease symptoms in the *Daphnia*, we did observe a reduced survival of *D. magna* when simultaneously exposed to both the parasite and the pesticide. No increased mortality upon exposure to individual stressors was observed. We did not observe an evolutionary change in fitness response of the *Daphnia* clones hatched from different time horizons upon exposure to stressors.

**Keywords** *Daphnia magna* · Diazinon ·
Dormant eggs · Multiple stressors · *Pasteuria ramosa* ·
Parasites · Resurrection ecology

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## Introduction

In recent years, the importance of multiple stressors on the health of organisms has received increased attention (e.g. Marcogliese and Pietrock 2011). It is well known that responding to additional disturbances leads to higher costs, which can result in a reduction of fitness of exposed individuals (reviewed in Relyea 2003; Sih et al. 2004). Anthropogenic stress is expected to often increase the incidence and impact of disease in natural populations (e.g. Daszak et al. 2000). Pesticides, for example, are used worldwide in agriculture and have been shown to increase

susceptibility to parasite infections, as shown for example for oysters (Chu and Hale 1994), amphibians (Gendron et al. 2003; Rohr et al. 2008; King et al. 2010), fish (Kreutz et al. 2010) and planktonic crustaceans (Coors et al. 2008). This synergistic effect may reflect immune suppression by the pesticide (Galloway and Handy 2003) or a cumulative effect of increased energy demand imposed by each of the stressors (Sheldon and Verhulst 1996).

In experimental studies on host–parasite interactions of animals and plants exposed to biotic and abiotic stressors, fitness consequences have often been measured for individuals originating from different locations (King et al. 2010; Schoebel et al. 2010; Bryner and Rigling 2011; Jansen et al. 2011). This approach provides valuable information about patterns of genetic adaptation to local environmental conditions (Kawecki and Ebert 2004). In order to study evolutionary responses to environmental changes, however, it would be necessary to directly track changes over time (De Meester et al. 2007). For such questions, "resurrection ecology" (bringing back ancestors genetic material for transgenerational tests; Kerfoot et al. 1999) can be a powerful tool (Hairston et al. 1999, 2001; Cousyn et al. 2001; Jeppesen et al. 2001; Kerfoot and Weider 2004). This approach is nicely applicable to aquatic organisms, as some species, for example the planktonic crustacean *Daphnia*, can produce dormant egg stages that can be preserved in the sediment (Carvalho and Wolf 1989; Cáceres 1998). From dated sediments, *Daphnia* genotypes that were produced at different points of time can be hatched and used in experiments. Several resurrection ecological studies have provided evidence for microevolutionary responses over only a few decades (e.g. Hairston et al. 1999, 2001; Cousyn et al. 2001). For example, Hairston et al. (1999), using *Daphnia* hatched from a period when toxic cyanobacteria were present in Lake Constance showed that they were more resistant to a diet with cyanobacteria, in comparison with *Daphnia* hatched from a period when no toxic cyanobacteria were present. Cousyn et al. (2001) documented microevolutionary changes in phototactic behaviour in *Daphnia* populations in response to changes in fish predation pressure, and Pauwels et al. (2010) showed that the same population also evolved with respect to phenoloxidase activity, a component of the invertebrate innate immunity. Finally, Decaestecker et al. (2007), using both

*Daphnia* and its microparasite *Pasteuria ramosa* hatched from different sediment layers, showed strong parasite–host co-adaptive responses.

In the present study, we tested whether the combined exposure to the parasite *Pasteuria ramosa* (Metchnikoff 1888) and the pesticide diazinon affected the fitness of *Daphnia magna* (Straus, 1820), hatched from different sediment layers. Whereas the use of diazinon over time is not recorded for the pond where the *Daphnia* were hatched from, earlier studies on the same population found density differences of *P. ramosa* spores between sediment layers (Decaestecker et al. 2004, 2007). The parasite abundance can influence the host's investment in immunological defences (Schmid-Hempel and Ebert 2003; Lindström et al. 2004); therefore, we expect that hosts hatched from layers with high parasite abundance get less infected than those with lower parasite abundance. It also has been shown that immune defences are coupled with costs (Sheldon and Verhulst 1996; Norris and Evans 2000) and that combined stress can increase infection rates (e.g. Coors et al. 2008). For example, a reinforcement of parasite infection rate in the presence of the organophosphate carbaryl has been shown (Coors and De Meester 2008, 2011; Coors et al. 2008). We expect that the effects of an organophosphate pesticide on *Daphnia* hatched from sediment layers with high parasite densities may be stronger compared to its effects on *Daphnia* hatched from parasite-poor sediment layers.

## Materials and methods

### Parasite and pesticide used as stressors

As parasite stress, we used the horizontally transmitted *Pasteuria ramosa* (Metchnikoff 1888; Ebert et al. 1996; Ebert 2005), a gram-positive bacterium co-occurring with *Daphnia magna* in the field (Ebert 2005). Infected hosts become infertile, the body becomes darkish and grows large and are therefore easily recognizable by eye (Ebert 2005). *P. ramosa* spores, which are released after host death, are found in pond sediments and remain infective for decades (Decaestecker et al. 2004). As pesticide stress, we used the organophosphate diazinon (O,O-diethyl O-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphothionate). This pesticide was developed in 1952 and heavily used

between 1970 and the early 1980s in many parts of the world. Nowadays, diazinon is still frequently used in agriculture and households and can thus be detected in aquatic environments (Konstantinou et al. 2006; Singer et al. 2010; Wittmer et al. 2010). Acute toxic effects of diazinon (e.g. immobilization and death) are related to the inhibition of acetylcholinesterase, an enzyme essential for proper function of the nervous system (Chambers 1992; Maxwell et al. 2006). Exposure of *D. magna* to sublethal concentrations of diazinon leads to a delay in reproduction, a decrease in the number and size of offspring, and higher adult mortality (Sanchez et al. 1998).

### Origin and culture conditions of the host–parasite system

*D. magna* host clones were used from a previous study (Decaestecker et al. 2007). The hatched *Daphnia* came from seven different sediment layers of the Belgian pond OM2 situated in Heverlee (50°51′48″N, 4°43′16″E), covering a time period of about 17–28 years (Decaestecker et al. 2007). This pond is characterized by epidemics of *P. ramosa* (Decaestecker et al. 2007), as well as intense agricultural activity in the catchments area (Coors et al. 2009). In total, we used 13 genetically different clones, two from each sediment layer, except from the oldest, where only one clone could be used.

P. ramosa spores used for infection were obtained by exposing different individuals of a single *D. magna* clone (M10, see Cousyn et al. 2001) originating from the pond Oud Heverlee (Belgium, 50°50′22″N, 4°39′18″E) to the first few centimetres of the sediment core from pond Knokke Nat (Belgium, 51°21′25″N, 3°19′50″E), as described in Jansen et al. (2010). The parasites were collected from a different pond than the host clones used in the experiment to avoid possible effects of co-adaption of hosts and parasites (Decaestecker et al. 2007). To increase the amount of *P. ramosa* spores, infected M10 clones were grown for 21 days and used to infect *Daphnia* juveniles of the same clone for another generation, before using them in the experiment as described in Coors et al. (2008).

### Experimental set-up and procedures

Thirteen clones (from seven sediment layers) were exposed to *P. ramosa* and diazinon (CAS 333-41-5, 99.5 % purity, Ultra Scientific Analytical Solutions, N. Kingstown RI, USA) in a full factorial design and replicated six times, resulting in 312 experimental units. To minimize maternal effects at the start of the experiment, the clones were kept under standard experimental laboratory conditions [1:5 diluted Aachener Daphnien Medium (ADaM, Klüttgen et al. 1994), $20 \pm 2$ °C, 16 h:8 h light/dark photoperiod, fed daily with $2 \times 10^5$ *Scenedesmus obliquus* cells/mL] for at least two generations. To start the experiment, ten third-clutch juveniles (less than 24 h old) were put together in 250-mL glasses containing 20 mL of medium. The pesticide treatment consisted of a concentration of diazinon (0.25 µg/L), which is sublethal for *Daphnia* (Sanchez et al. 1998), whereas the pesticide-free control treatment included the addition of the same amount of the solvent (acetone 0.008 %). Concentrations of diazinon were verified with spot test with LC–MS/MS as described in Kretschmann et al. (2011) and were all within $\pm 20$ % of the nominal concentrations. Individuals belonging to the parasite treatment were exposed on day 0 and day 2 to $3.75 \times 10^4$ mature spores of *P. ramosa* per mL (Jansen et al. 2011). This spore solution was prepared 1 week prior to the experiment and frozen at $-21$ °C, a method successfully used in other experiments (M. Jansen, unpublished). All jars belonging to the control treatment were treated with ground-up, non-infected *Daphnia* of the M10 clone. On day 5, all *Daphnia* were transferred into new jars (500 mL) containing 400 mL medium, which was renewed every third day thereafter. Animals were checked for infections, the number of offspring was counted and the survival rate was checked daily (and the dead individuals removed) till the end of the experiment (day 21). All traits were quantified as a mean or proportion per set of 10.

### Statistical analyses

Statistical analyses were performed using SPSS 19.0. In total, seven experimental units were lost due to handling errors and excluded from the analysis. Differences in survival rate and reproductive output were analysed using linear mixed effects models, with exposure to parasite (yes/no), exposure to diazinon (yes/no) and sediment layer as fixed factors and clone nested in sediment layer as a random factor. We did not include the interactions between the fixed factors and the random factor clone, as our aims were not

related to clonal variation. The individual reproductive output was calculated (total amount of offspring born on day x divided by the total amount of living females on day x, for x going from day 4 to 21; Van Doorslaer et al. 2009). All residuals were distributed normally.

## Results

In this experiment, no visible signs of infections could be observed at day 21. Nevertheless, significant differences in survival rate were observed when *Daphnia* were exposed to both stressors: The proportion of surviving individuals was significantly reduced when *Daphnia* were exposed to parasite spores and the pesticide simultaneously, but not when they were exposed to a single stressor (Table 1; Fig. 1). For individual reproductive output, there was no significant effect of these factors nor of their interactions (Table 1). Further, there were no interaction effects with the sediment layer, nor a main effect of the sediment layer on any of our response variables (all $p > 0.1$, see Table 1; Figs. 2, 3). All measured traits were highly variable among clones (Figs. 2, 3).

## Discussion

Our key result is the increased mortality of *D. magna* upon exposure to both a parasite and a pesticide, even though no parasite infections could be detected
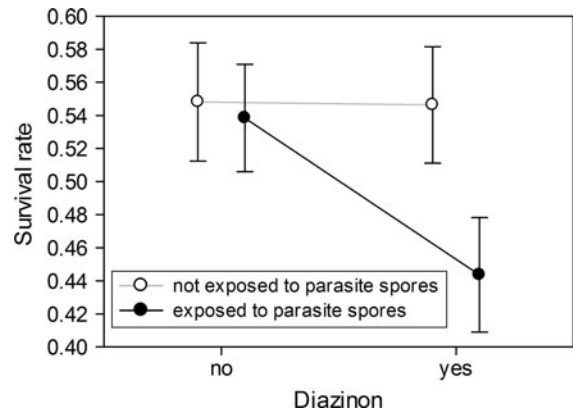


**Fig. 1** Mean proportion of surviving individuals per jar (±SE) as a function of exposure to the pesticide diazinon (no/yes) and parasite spores (no = *open circles*/yes = *closed circles*)

visually (see further). Many studies showed that the negative effect of two stressors on survival is much stronger compared to individual effects. For example, hosts in poor condition have higher parasite-induced mortality than hosts in good condition (e.g. Braune and Rolff 2001; Krist et al. 2004; Jokela et al. 2005). Immune suppression under direct exposure to pollutants is a well-known phenomenon (e.g. McDowell et al. 1999; Aggarwal et al. 2008), and also diazinon is known to have immunomodulatory effects (Galloway and Handy 2003; Oostingh et al. 2009; Holmstrup et al. 2010). Our results indicate that the defence against parasite spores, even if they do not cause a visible infection, is associated with costs for

**Table 1** Results of linear mixed effects models describing the influence of exposure to parasite spores, exposure to diazinon, sediment layer and interactions among these factors on survival and reproduction

| Dependent variable | Source | Numerator $df$ | Denominator $df$ | $F$ value | $p$ value |
|---|---|---|---|---|---|
| Survival rate | Parasite (P) | 1 | 271.03 | 3.01 | 0.084 |
| | Diazinon (DZ) | 1 | 271.03 | 2.88 | 0.091 |
| | Sediment layer (S) | 6 | 6.04 | 1.93 | 0.220 |
| | P × DZ | 1 | 271.09 | 5.10 | 0.025 |
| | P × S | 6 | 271.04 | 0.69 | 0.659 |
| | DZ × S | 6 | 271.03 | 1.60 | 0.147 |
| | P × DZ × S | 6 | 271.09 | 0.98 | 0.438 |
| Reproductive output | Parasite (P) | 1 | 267.06 | 0.87 | 0.351 |
| | Diazinon (DZ) | 1 | 267.14 | 0.28 | 0.597 |
| | Sediment layer (S) | 6 | 6.03 | 2.19 | 0.180 |
| | P × DZ | 1 | 267.15 | 0.23 | 0.629 |
| | P × S | 6 | 267.07 | 1.04 | 0.398 |
| | DZ × S | 6 | 267.15 | 1.64 | 0.136 |
| | P × DZ × S | 6 | 267.16 | 1.32 | 0.248 |

**Fig. 2** Mean proportion of surviving individuals per jar (±SE) for each clone per sediment layer (from deepest (7) to newest (1) sediment layer) as a function of exposure to the pesticide diazinon (no = circles/ yes = squares) and parasite spores (no = white/ yes = black)
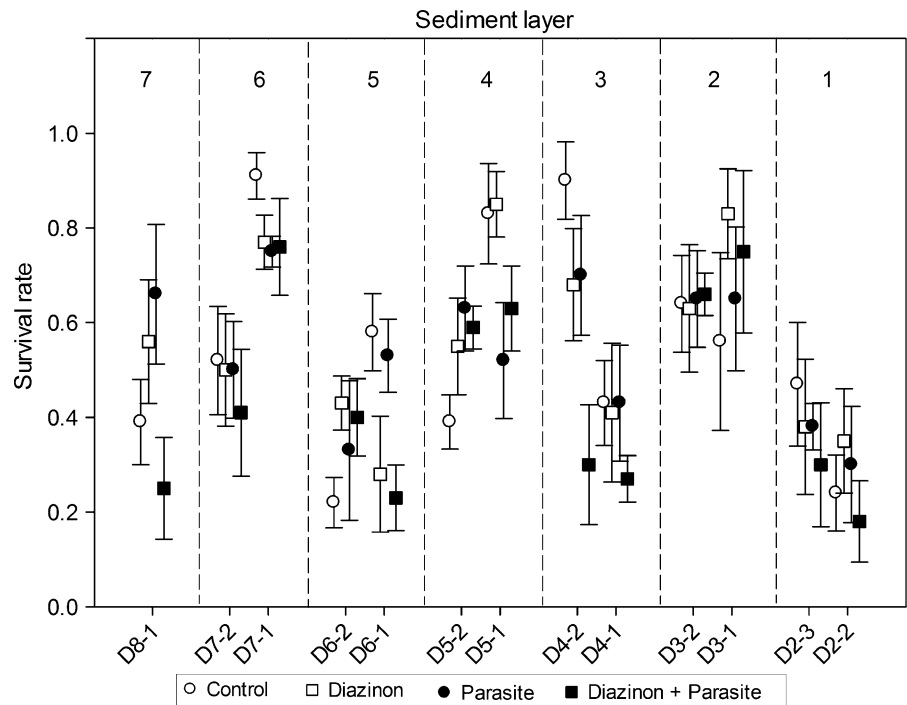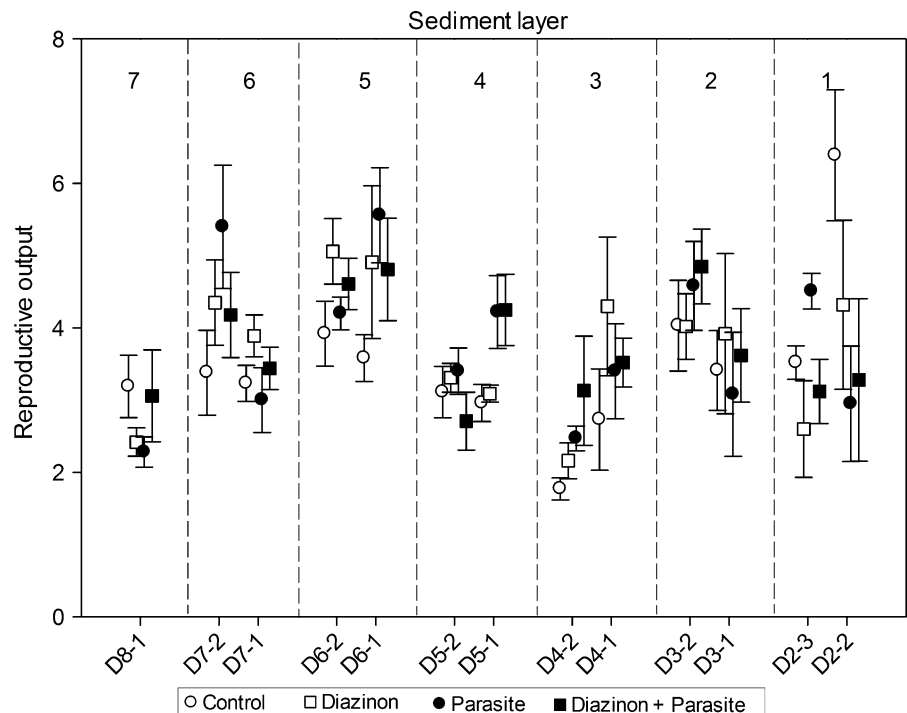


**Fig. 3** Mean amount of offspring per female per day (reproductive output; ±SE) for each clone per sediment layer (from deepest (7) to newest (1) sediment layer) as a function of exposure to the pesticide diazinon (no = circles/ yes = squares) and parasite spores (no = white/ yes = black)



*D. magna*. Another study also found costs of defences in *Daphnia*. Yin et al. (2011) showed that defence against predators induced stronger disease progression in *Daphnia*. Here we show that the enhanced costs of defending against parasite spores, even if they do not cause visible infections, increase mortality upon

exposure to a pesticide. Our findings agree with the study of Coors et al. (2008), who showed that exposure to pesticides increased disease progression in *Daphnia* and with other studies that show an association between energy cost and immune response in this system (Little and Killick 2007; Labbe et al. 2010; Allen and Little 2011). However, we cannot explain the generally high mortality rate in our control treatment.

There are three potential reasons for why the *D. magna* clones in our study did not get infected by *P. ramosa*. First of all, it is possible that either during parasite isolation from the sediment or during enrichment of parasite spores, M10 clones were infected by parasite spores that were not infective for OM2 clones, even though *P. ramosa* from Knokke Nat have been shown to heavily infect *D. magna* clones from OM2 (Jansen et al. 2010). Second, it is possible that our additional round of infection of clone M10 resulted in a bottleneck with respect to the number of parasite lineages and that a strain was selected which by chance did not infect OM2 clones. Alternatively, it may be that, for unknown reasons, the number of viable *P. ramosa* spores decreased to below-threshold levels in the time between estimating their density and using them in the experiment. Low spore concentrations result in failure to induce disease (Ben-Ami et al. 2008). The observed increased mortality in our combined parasite × pesticide exposure treatment may thus be associated with either exposure to a below-threshold concentration of parasite spores or exposure to spores that were not infective.

We did not find support for our hypothesis that *Daphnia* hatched from sediment layer with higher spore densities can cope better with parasite infections. No effect of sediment layer on any measured trait was observed, which is in contrast to Decaestecker et al. (2007), who found a correlation between infectivity and year (represented by the different layers here) as well as with the study of Pauwels et al. (2010), who found differences in levels of *Daphnia* phenoloxidase activity over time. Unfortunately, while our parasite exposure treatment clearly elicited a significant effect in terms of mortality, the absence of visual signs of infection warns against overinterpretation of our lack of a differential response for *Daphnia* isolated from different sediment layers. If the exposure was successful in generating a stress response or boost in immune system functioning, but

not strong enough to cause any infections, our results do not allow us to test for interaction effects between sediment layer (exposure to parasites in natural habitat) and infection dynamics. So while our results reveal an interesting effect of combined exposure on mortality, they may not provide a good test for our basic hypothesis.

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