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# 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-senstive (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 °C ± 1 °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 $2 \times 10^5$ 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°50′22″N, 4°39′18″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 °C ± 1 °C). The medium was renewed every other day. Temperature (mean ± SD: 19.3 ± 0.4 °C), oxygen concentration (mean ± SD: 9.05 ± 0.27 mg $L^{-1}$), pH (mean ± SD: 7.63 ± 0.10) and conductivity (mean ± SD: 892 ± 20 μS $cm^{-1}$) 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°20′6″N, 3°20′54″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 μm nylon filter (Millipore) and then diluted with deionized water to a concentration of $5 \times 10^6$ spores $mL^{-1}$. A placebo-suspension for parasite-free treatments was prepared in the same way by grounding 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× 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 $3.75 \times 10^4$ 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

placebo solution. No spores or placebo-solution were added to the medium later on in the experiment.

*2.3.2. Pesticide challenge*

Daphnids were challenged with 8 $\mu g\,L^{-1}$ carbaryl (1-naphthyl methylcarbamate, 99.8% purity, Sigma–Aldrich) during the first six days of the exposure. Carbaryl was added to fresh medium at the start of the experiment (day 0) and at the time of the media renewals. The US Environmental Protection Agency report on the ecological risk assessment of carbaryl reports measured surface water concentrations of carbaryl up to 5.5 $\mu g\,L^{-1}$ and estimated peak concentrations ranging between 23 and 153 $\mu g\,L^{-1}$ (US EPA, 2003), pointing to the environmental relevance of the carbaryl concentration used in the present study. Carbaryl stock solutions were prepared in ethanol and the ethanol concentration in the exposure was set to the same level in all treatments, including treatments without carbaryl (50 $\mu L\,L^{-1}$). Three mixed samples of 250 mL of each fresh and 48-h old medium (i.e. sample taken immediately after transferring daphnids to fresh media) separately were taken and stored in brown glass bottles at −20 °C for later verification of carbaryl concentrations. Analysis of carbaryl concentrations was done by GC–MS (Trace GC 2000 series, Thermoquest; Polaris, Finnigan/Thermoquest) on an apolar SLB$^{TM}$-5ms column (Supelco, Sigma–Aldrich). Extraction and elution was performed on Solid Phase Extraction according to the manufacturer's application notes (Waters and Phenomenex). Propoxur was used as the internal standard at a concentration of 4 $\mu g\,L^{-1}$ to control and correct for extraction losses. Recovery was always >90%. Proxopur belongs to the same functional class of pesticides as carbaryl, i.e. the carbamates. Immediately before injection of the sample, a recovery standard was also applied, to control for the injection itself. The carbaryl concentration was $8.85 \pm 0.21$ $\mu g\,L^{-1}$ (mean ± standard deviation) in freshly prepared medium and $6.57 \pm 0.40$ $\mu g\,L^{-1}$ in 48-h old medium (immediately after renewal).

*2.4. Life-history endpoints*

Maturation rate is reported as the percentage of egg-carrying individuals on day 8 of the exposure. No offspring were released from the brood pouches before this day in none of the experimental cultures. Investment in early reproduction is reported as the number of offspring produced between media renewals on day 8 and day 10 divided by the number of egg-carrying individuals counted on day 8. Body length on day 10 of six to eight animals was measured from the top of the head to the base of the spine by analyzing a microscopic image with the Image Tool 3.0 software (San Antonio, TX, USA).

*2.5. Physiological endpoints*

*2.5.1. Feeding rate*

Filtration rate was measured at the end of the experiment (day 10) according to the method described in Muyssen et al. (2006) with minor modifications. Three replicates of one individual daphnid per treatment were set up and three 'blancs' without daphnids (but with algal food added) per treatment were used to be able to account for algal growth when calculating filtration rate. The algal concentrations (*P. subcapitata*) were measured using a Coulter Counter (Z1 Coulter Particle Counter, Beckman Coulter) at the beginning of the feeding period and after 24 h.

*2.5.2. Energy reserves and electron transport system activity*

Energy reserves were measured on day 10 as three separate energy fractions: protein, lipid and carbohydrate content of the organisms. For each fraction seven daphnids were collected and flash-frozen in liquid nitrogen on day 10. Samples were stored at −80 °C until analysis. The different fractions were measured spectrophotometrically in triplicate and transformed into energetic equivalents as described in De Coen and Janssen (1997). The energy consumption was estimated by measuring the electron transport system (ETS) activity at the mitochondrial level as described in De Coen and Janssen (1997). ETS activity was measured as an alternative to oxygen consumption measurements as these could not be performed due to a broken probe.

*2.5.3. Acetylcholinesterase activity*

Pools of seven flash-frozen daphnids collected on day 10 were homogenized in 0.02 M ice-cold sodium hydrogen phosphate buffer (PB), pH 8.0, containing 1% Triton-X-100 (Sigma–Aldrich) with a motor-driven Teflon pestle for 45 s. Ice-cold PB (without Triton-X-100) was added to the initial homogenate in a 10:1 ratio. The final homogenates were mixed and centrifuged at $3000 \times g$ at 2–4 °C for 10 min. Supernatants were collected in a clean, pre-cooled Eppendorf tube and assayed immediately. The enzyme activity was determined in triplicate for each sample according to the colorimetric method described by Ellman et al. (1961). Briefly, 100 μL of 8 mM 5-5′-dithiobis-2-nitrobenzoate (DTNB) (Sigma–Aldrich) in PB supplemented with sodium hydrogen carbonate (Sigma–Aldrich) at 0.75 $mg\,mL^{-1}$ and 50 μL of supernatant were added to a 96-well microtiter plate. Measurement of enzyme activity was initiated by adding 50 μL of 16 mM acethylthiocholine iodide (Sigma–Aldrich) in PB. Spontaneous hydrolysis of the substrate was assessed using a blank in triplicate, containing PB with 0.1% Triton-X-100 instead of the supernatant. After an incubation period of 10 min at 20 °C, absorbances at 405 nm and 20 °C were measured every 60 s during 10 min with intermittent shaking.

The enzyme activity was expressed in $nmol\,min^{-1}\,mg^{-1}$ as activity = $(\Delta OD/min)/(\varepsilon \times l \times C)$ where $\Delta OD$/min is the change in optical density per minute ($min^{-1}$), $\varepsilon$ is the molar extinction coefficient of DTNB ( = $1.34 \times 10^6$ $nM^{-1}cm^{-1}$), $l$ is the length of the light path (cm), $C$ the protein concentration in the supernatans ($mg\,L^{-1}$).

Protein concentration in the homogenate supernatant was determined using the Bradford method (Bradford, 1976), with bovine serum albumin (Sigma–Aldrich) as a standard.

Quality control of the assay was assessed using a quality control enzyme standard of electric eel cholinesterase (Sigma–Aldrich) in ice-cold PB containing 1 $mg\,mL^{-1}$ bovine serum albumin (Sigma–Aldrich). The reaction rate of the quality control enzyme was confirmed at a change of 55–60 $mOD\,min^{-1}$.

*2.5.4. Phenoloxidase activity*

A major component of the invertebrate innate immune system is the prophenoloxidase (proPO) activation system, providing immunity against a large range of pathogens (Söderhall and Cerenius, 1998; Cerenius et al., 2008). Upon infection, the inactive proenzyme proPO is activated and transformed into the active form phenoloxidase (PO), which oxidizes phenols and thus leads to the formation of melanin, which is believed to play an important role in encapsulation and neutralization of bacteria (Söderhall and Cerenius, 1998).

Measurement of phenoloxidase (PO) activity normally uses extracted haemolymph as described by Mucklow and Ebert (2003). However, because carbaryl-treated daphnids of clone S at the end of the exposure were too small to extract sufficient amounts of haemolymph, we chose to use real-time qPCR analysis of the proPO gene as an alternative. We used the method described by Labbé and Little (2009). Daphnids collected on day 10 were shock-frozen in liquid nitrogen prior to total RNA isolation. Total RNA isolation was performed using RNeasykit and Qiashredder kit (Qiagen) following manufacturer's instructions. Contaminating DNA was removed by a DNAse treatment (Qiagen). Prior to cDNA transcription, RNA quality and quantity were determined with a Nanodrop spectrophotometer. RNA aliquots for reverse transcriptase were stored at −80 °C and

afterwards reverse transcribed to cDNA using 1 μg of RNA and the MessageAmpTM II mRNA Amplification kit (Applied Biosystems) according to manufacturer's protocol. Only first strand cDNA synthesis was performed. Sample quality and yield were again assessed using the Nanodrop spectrophotometer. Samples were stored at −20 °C until qPCR analysis, which was performed on a Corbett RotorGene 3000 during 45 cycles (30 s at 95 °C; 30 s at 58 °C; 35 s at 72 °C). Further qPCR analysis was performed as described in Labbé and Little (2009).

### 2.6. Data treatment and statistical analyses

In all statistical tests performed, all data were balanced, i.e. an equal number of replicate observations was available for each treatment for each endpoint. All statistics were performed with Statistica 7.0 software (Statsoft, Tulsa, OK, USA). All endpoints were $\log_{10}$-transformed prior to statistical analysis to ensure compliance with assumptions of normality (Shapiro–Wilkinson's $W$ test) and homoscedasticity (Levene's test) for all endpoints. This transformation also allowed us to interpret findings of a statistically significant carbaryl × parasite interaction term in a two-way ANOVA as a statistically significant deviation from the independent action (IA) model of joint stressor effects (Sih et al., 1998; Fournier et al., 2006; see further).

First, we performed three-way ANOVA to determine the significance of the main effects and two-way and three-way interaction terms for all endpoints. All analyses were performed at a significance level of 95% ($p < 0.05$). Of particular interest were findings of significant clone × carbaryl interaction (confirming a different effect of carbaryl between the two clones) and significant three-way clone × carbaryl × parasite interaction. While three-way interactions can be interpreted in different ways (Kutner et al., 2005) one possible interpretation in our study is that it indicates that the carbaryl × parasite interaction is different between the two clones, which is exactly what we wanted to test in relation to the aims of our study. Therefore we also performed a more detailed analysis of the carbaryl × parasite interaction with two-way ANOVA's for each clone separately to aid the validation of such an interpretation (e.g., if this interaction would be significant in one clone but not in the other). At the same time, the same two-way ANOVA analysis provided a formal statistical test of the independent action (IA) model (see below).

Second, we investigated for each endpoint and for each clone separately if the effect observed in the combined P + C treatment followed the Independent Action (IA) model. This is the recommended reference model for predicting combined effects of dissimilarly acting stressors (Jonker et al., 2004) and thus the logical choice in the present study based on the biologically fundamentally different mechanisms of action of carbaryl and *Pasteuria* infection in *Daphnia* (Coors et al., 2008). This model, originally formulated by Bliss (1939), predicts combined effects of binary stressors from observed effects in the individual stressor treatments as follows (Faust et al., 2003):

$$E_{\text{PC, predicted}} = E_{\text{P}} + E_{\text{C}} - E_{\text{P}} \times E_{\text{C}} \quad (1)$$

where

$$E_i = \frac{Y_{\text{control}} - Y_i}{Y_{\text{control}}} \quad (2)$$

with $E_i$ the observed fractional effect of treatment $i$ on endpoint $Y$ relative to the control treatment, where $i$ is either P (*Pasteuria*), C (carbaryl) or PC (combined *Pasteuria* + carbaryl treatment). It should be noted that $E_i$ can be both positive (in case of a decrease of the endpoint compared to the control) and negative (in case of an increase of the endpoint compared to the control). Algebraically combining Eq. (1) and Eq. (2), also allows predictions of the value of each endpoint in the combined carbaryl + parasite treatment ($Y_{\text{PC}}$, depicted in Figs. 1 and 2), based on the arithmetic mean of the values observed in the control ($Y_{\text{control}}$), the carbaryl only treatment ($Y_{\text{C}}$) and the parasite only treatment ($Y_{\text{P}}$):

$$Y_{\text{PC, predicted}} = \frac{Y_{\text{P}} \times Y_{\text{C}}}{Y_{\text{control}}} \quad (3)$$

The actual statistical testing of the hypothesis of independent action for each clone separately was implemented by determining the significance of the *Pasteuria* × carbaryl interaction term in the 2-way ANOVA's carried out on $\log_{10}$-transformed observational data for each clone separately. In other words, a significant interaction term at the 95% significance level ($p < 0.05$) found with this ANOVA implies a statistically significant deviation from IA. This approach has infrequently been used in the field of ecotoxicology for testing deviations from the IA model for binary chemical stressor mixtures. In contrast, it is already being used for more than a decade in the field of ecology for detecting significant departures from independent action of binary combinations of ecological stressors, for instance prey stressed by two predators (review by Sih et al., 1998) or plants stressed by two parasites (Fournier et al., 2006).

Third, when the 2-way ANOVA revealed a statistically significant *Pasteuria* × carbaryl interaction, we classified it as synergistic if the observed effect in the combined treatment was 'higher' than the effect predicted with the IA model (Eq. (1)) (Faust et al., 2003). In terms of the calculations made with Eq (1) and Eq (2)., this occurs if $E_{\text{PC, observed}} > E_{\text{PC,predicted}}$ in cases where $E_{\text{PC, observed}} > 0$ (i.e., where the combined treatment causes a reduction of the endpoint compared to the control, e.g. survival, see Fig. 1A) or if $E_{\text{PC, observed}} < E_{\text{PC, predicted}}$ in cases where $E_{\text{PC,observed}} < 0$ (i.e., where the combined treatment causes an increase of the endpoint compared to the control, e.g. proPO expression, see Fig. 2G). When the observed effect was 'smaller' than the predicted effect, i.e. if $E_{\text{PC, observed}} < E_{\text{PC, predicted}}$ in cases where $E_{\text{PC, observed}} > 0$, or if $E_{\text{PC,observed}} > E_{\text{PC, predicted}}$ in cases where $E_{\text{PC, observed}} < 0$, the interaction was classified as antagonistic.

## 3. Results

Results for all measured endpoints are presented in Figs. 1 and 2. Results of three-way ANOVA analyses are given in Tables 1 and 2 for life-history and physiological endpoints, respectively. Tables 1 and 2 also contain results of the two-way ANOVA analysis of the parasite × carbaryl interaction for clones NS and S separately. Complete two-way ANOVA results are listed in Tables S1 and S2 in supplementary material.

Main effects of clone, parasite and carbaryl were detected in most endpoints, with few exceptions (Tables 1 and 2). Sterilization already reached 100% on day 10 in all parasite and parasite + carbaryl treatments in both clones, thus making any further testing of parasite × carbaryl interactions impossible for this endpoint.

### 3.1. Life history endpoints

With three-way ANOVA, significant clone × carbaryl interactions were observed for all life-history endpoints, showing stronger reductions of survival, investment in early reproduction, body length and maturation rate after carbaryl exposure in the carbaryl sensitive clone S than in the non-sensitive clone NS (Fig. 1, Table 1). No significant clone × parasite interactions were observed for any of the life-history endpoints (Table 1). Our observations on the variables scored indicate that the two studied clones show differences in their sensitivity towards carbaryl but not towards the parasite.

Significant three-way clone × carbaryl × parasite interactions suggest that there are clonal differences in carbaryl × parasite

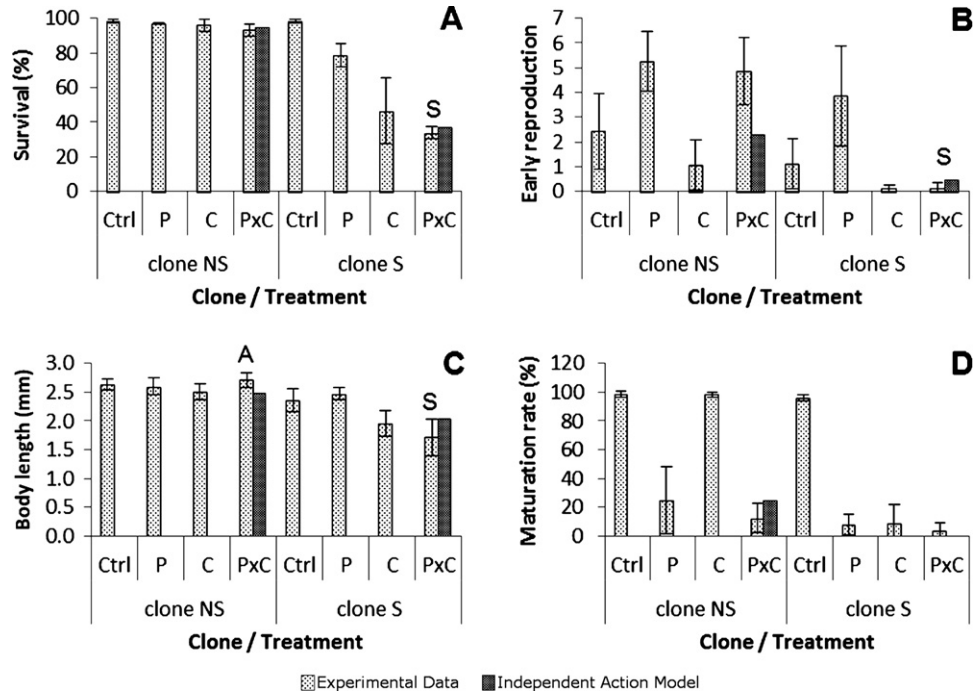


**Fig. 1.** Life-history endpoints (A: survival; B: early reproduction; C: body length and D: maturation rate) for clones NS (carbaryl non-sensitive) and S (carbaryl sensitive) for all treatments. Predicted values of each endpoint for the combined treatment according to the Independent Action Model, calculated with Eq. (3), are also depicted. (A/S) indicates significant antagonistic and synergistic interactions, respectively. Early reproduction is expressed as the number of juveniles per egg-carrying individual on day 8 and maturation rate as the percentage egg-carrying individuals on day 8. Ctrl: control; P: parasite exposure; C: carbaryl exposure; PxC; combined parasite and carbaryl exposure. Error bars indicate standard deviation.

interactions for three of the four tested life-history endpoints (i.e. survival, investment in early reproduction and body length) (Table 1, Fig. 1A–C). No significant three-way interaction was observed for maturation rate (Table 1, Fig. 1D). Detailed follow-up analyses of the three significant three-way interactions with two-way ANOVA indicated that no interactive effect between parasite and carbaryl on survival was found for clone NS, while a synergistic interaction was detected for clone S (Table 1; Fig. 1A). Clone S also showed a synergistic parasite × carbaryl interaction effect for early reproduction, while clone NS did not (Table 1, Fig. 1B). Finally, for body length an antagonistic interaction was observed for clone NS (Table 1, Fig. 1C), while a synergistic interaction was detected for clone S (Table 1, Fig. 1C).

### 3.2. Physiological endpoints

With three-way ANOVA, differences in response to carbaryl among the two clones were observed for some measured physiological endpoints, with significant clone × carbaryl interaction terms for protein and carbohydrate reserves (Table 2). While carbaryl has no effect in clone NS, it has a strong negative effect on the total protein and carbohydrate reserves in clone S (Fig. 2B and C). In addition, two-way ANOVA revealed significant clone × carbaryl interactions in the absence of parasites for ETS ($p < 0.001$) and AChE ($p = 0.01$). For the latter two endpoints carbaryl has a strong positive effect in clone S, while no effect was detected in clone NS (Fig. 2E and F). No clone × carbaryl interaction was detected with three-way ANOVA for filtration rate (Fig. 2A), lipid reserves (Fig. 2D) and proPO expression (Fig. 2G). Together, our observations indicate that carbaryl elicits a very different response of the physiological endpoints studied in both clones, with an overall more pronounced effect in clone S. None of the measured physiological endpoints showed significant three-way interactions. However, in two-way ANOVAs carried out for both clones separately, a significant, antagonistic parasite × carbaryl interaction was detected in clone NS for the proPO expression endpoint, while no interaction was detected for clone S (Table 2). This suggests a tendency for differences in the response to a combined effect of parasite and carbaryl exposure between these two clones for this endpoint. No carbaryl × parasite interactions were observed with two-way ANOVA for ETS and AChE in clone S, while a significant, antagonistic carbaryl × parasite interaction was detected for ETS in clone NS.

## 4. Discussion

Susceptibility to the adverse effects of parasite infection can increase with host environmental stress (Gèrard et al., 2008). Evidence of chemical stressors interacting with parasites is mounting, but mostly limited to vertebrate species (Holmstrup et al., 2010). Kramarz et al. (2007) showed that the snail *Canthareus aspersus* exposed simultaneously to cadmium and the nematode *Phasmarhabditis hermaphrodita* accumulated cadmium to higher concentrations than control snails. Scarab grubs (*Cyclocephala hirta* and *C. pasadenae)* exposed to a combination of a biopesticide and nematodes showed additive or greater than additive mortalities (Koppenhöffer and Kaya, 1997). Synergistic interactions are also reported for the pesticide imidacloprid applied together with entomopathogenic nematodes in white grubs (*C. hirta*, *C. borealis* and *Popillia japonica*) in (Koppenhöffer et al., 2000). Finally, Cuthbertson et al. (2003) showed increased mortality of sweet potato whitefly larvae after exposure to a combination of imidacloprid and the nematode *Steinernema feltiae*. However, none of these studies investigated possible genotype-based differences in chemical × pathogen interactions between different genotypes of the same species that differ in their sensitivity to one of the stressors. One study by Salice and Roesijadi (2002) points in the direction of such differences: they found higher mortality due to cadmium in a parasite-resistant strain of the freshwater snail *Biomphalaria glabrata* compared to a parasite-susceptible strain. Yet, these authors did not expose both strains to a combination of

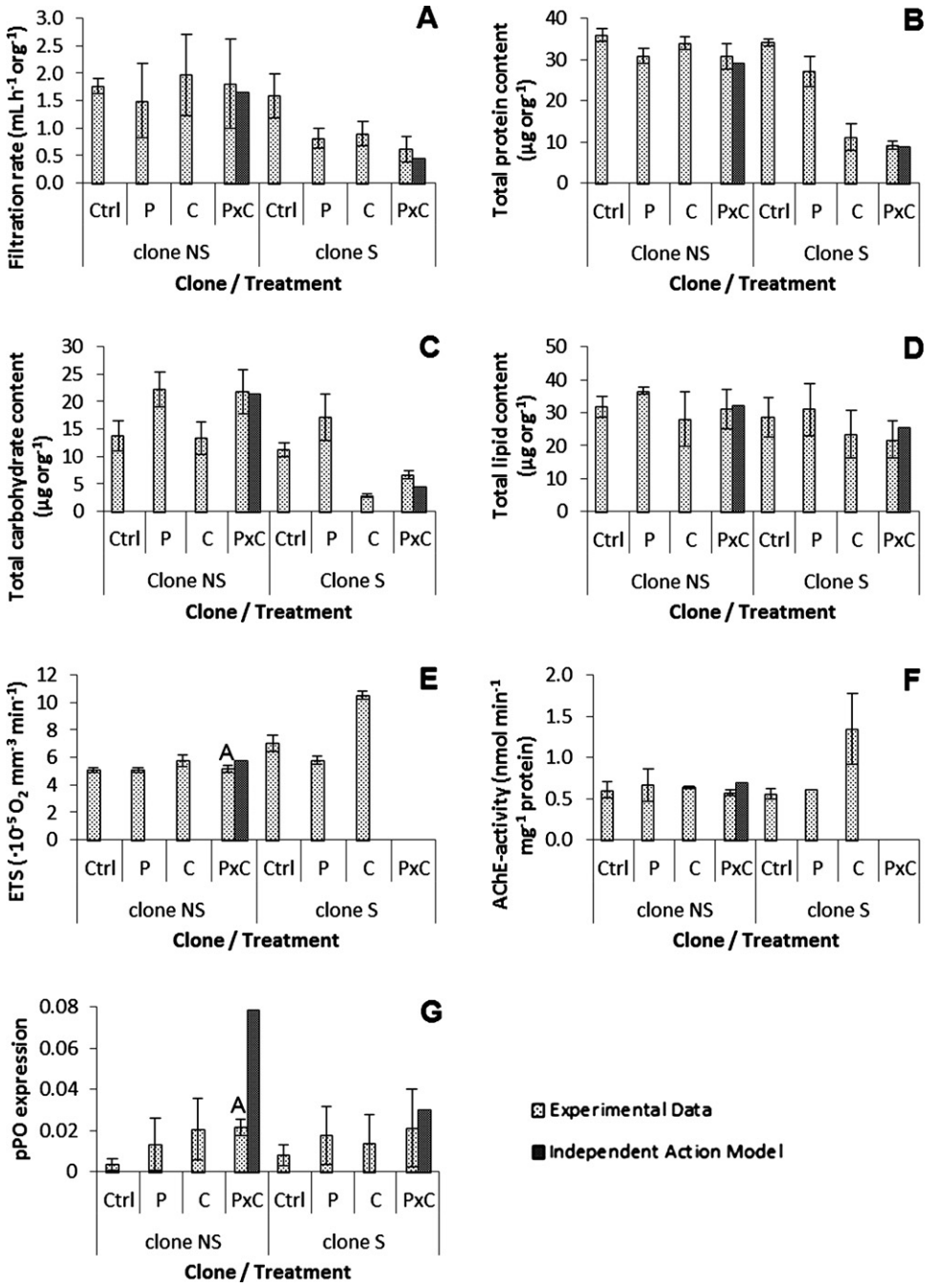


**Fig. 2.** Physiological endpoints (A: filtration rate; B: total protein content; C: total carbohydrate content; D: total lipid content; E: electron transport system activity; F: acetylcholinesterase activity and G: prophenoloxidase expression) for clones NS (carbaryl non-sensitive) and S (carbaryl sensitive) for all treatments. Predicted values of the endpoints in the combined treatment according to the Independent Action Model, calculated with Eq. (3), are also depicted. (A/S) indicates significant antagonistic and synergistic interaction, respectively. Ctrl: control; P: parasite exposure; C: carbaryl exposure; PxC; combined parasite and carbaryl exposure. Error bars indicate standard deviation.

cadmium and the parasite, making the assessment of interactions between both stressors impossible.

To be able to test whether the carbarayl-sensitive clone would experience different interactive effects between the insecticide carbaryl and the bacterial parasite *P. ramosa* compared to the less sensitive clone, we needed to verify first if one clone was indeed more sensitive to carbaryl than the other and whether both clones were overall equally sensitive to the parasite. For life-history endpoints, the three-way ANOVA showed clear clone × carbaryl interactions (Table 1). Clonal differences of daphnids in sensitivity to various stressors such as pesticides have been shown before (e.g. Calow et al., 1990; Warming et al., 2009). Both clones investigated here are affected by the parasite, with no significant clone × parasite interaction for the studied life-history endpoints (Three-way ANOVA, Table 1). Maturation rate decreased, while 'investment in early reproduction' increased following parasite exposure in both clones (Fig. 1B and D). In other words, fewer animals reached maturity in the parasite treatment after 8 days of exposure (compared to the control), but those that did reach maturity produced more juvenile offspring per animal on average. The observed decrease in maturation rate is most likely a direct effect of the parasite infection process (Ebert, 2005), while

**Table 1**
Summary of three-way ANOVA and two-way ANOVA for the two clones for life-history endpoints. Significant *p*-values at the 95% significance level are shown in bold. If significant parasite × carbaryl interactions were detected for a clone, the observed effect in the combined treatment (Eq. (2)), the predicted effect based on the Independent Action model (Eq. (1)) and the interaction type are also provided.

| | df | MS | *F* | *p* | Observed effect $E_{PC}$ | Predicted effect $E_{PC,\ predicted}$ | Interaction type |
|---|---|---|---|---|---|---|---|
| Survival | | | | | | | |
| *Three-way ANOVA* | | | | | | | |
| Clone | 1 | 6.261 | 52.904 | **<0.001** | | | |
| Parasite | 1 | 4.034 | 15.797 | **0.001** | | | |
| Carbaryl | 1 | 1.870 | 34.082 | **<0.001** | | | |
| Clone × Parasite | 1 | 0.217 | 1.835 | 0.194 | | | |
| Clone × Carbaryl | 1 | 1.915 | 16.180 | **<0.001** | | | |
| Parasite × Carbaryl | 1 | 0.267 | 2.255 | 0.153 | | | |
| Clone × Parasite × Carbaryl | 1 | 0.569 | 4.809 | **0.043** | | | |
| Error | 16 | 0.118 | | | | | |
| *Two-way ANOVA for the two clones* | | | | | | | |
| Parasite × Carbaryl at the level of Clone NS | | | | 0.696 | | | |
| Parasite × Carbaryl at the level of Clone S | | | | **0.007** | 0.66 | 0.62 | synergism |
| Early reproduction | | | | | | | |
| *Three-way ANOVA* | | | | | | | |
| Clone | 1 | 1.048 | 32.628 | **<0.001** | | | |
| Parasite | 1 | 0.673 | 20.279 | **<0.001** | | | |
| Carbaryl | 1 | 0.854 | 25.705 | **<0.001** | | | |
| Clone × Parasite | 1 | 0.083 | 2.509 | 0.133 | | | |
| Clone × Carbaryl | 1 | 0.277 | 8.355 | **0.011** | | | |
| Parasite × Carbaryl | 1 | 0.016 | 0.469 | 0.503 | | | |
| Clone × Parasite × Carbaryl | 1 | 5.901 | 5.901 | **0.027** | | | |
| Error | 16 | 0.033 | | | | | |
| *Two-way ANOVA for the two clones* | | | | | | | |
| Parasite × Carbaryl at the level of Clone NS | | | | 0.254 | | | |
| Parasite × Carbaryl at the level of Clone S | | | | **0.043** | 0.88 | 0.58 | synergism |
| Body length | | | | | | | |
| *Three-way ANOVA* | | | | | | | |
| Clone | 1 | 0.152 | 87.289 | **<0.001** | | | |
| Parasite | 1 | 0.0002 | 0.122 | 0.721 | | | |
| Carbaryl | 1 | 0.065 | 37.459 | **<0.001** | | | |
| Clone × Parasite | 1 | 0.005 | 3.009 | 0.088 | | | |
| Clone × Carbaryl | 1 | 0.063 | 36.058 | **<0.001** | | | |
| Parasite × Carbaryl | 1 | 0.002 | 1.076 | 0.304 | | | |
| Clone × Parasite × Carbaryl | 1 | 0.015 | 8.838 | **0.004** | | | |
| Error | 58 | 0.002 | | | | | |
| *Two-way ANOVA for the two clones* | | | | | | | |
| Parasite × Carbaryl at the level of Clone NS | | | | **0.041** | −0.03 | 0.06 | antagonism |
| Parasite × Carbaryl at the level of Clone S | | | | **<0.001** | 0.28 | 0.14 | synergism |
| Maturation Rate | | | | | | | |
| *Three-way ANOVA* | | | | | | | |
| Clone | 1 | 2.680 | 13.132 | **0.002** | | | |
| Parasite | 1 | 3.887 | 19.045 | **<0.001** | | | |
| Carbaryl | 1 | 1.685 | 8.255 | **0.011** | | | |
| Clone × Parasite | 1 | 0.035 | 0.173 | 0.683 | | | |
| Clone × Carbaryl | 1 | 0.913 | 4.474 | **0.050** | | | |
| Parasite × Carbaryl | 1 | 0.251 | 1.228 | 0.284 | | | |
| Clone × Parasite × Carbaryl | 1 | 0.701 | 3.436 | 0.082 | | | |
| Error | 16 | 0.204 | | | | | |
| *Two-way ANOVA for the two clones* | | | | | | | |
| Parasite × Carbaryl at the level of Clone NS | | | | 0.448 | | | |
| Parasite × Carbaryl at the level of Clone S | | | | 0.231 | | | |

df: degree of freedom; MS: mean square; *F*: *F*-value; *p*: *p*-value; IA: independent action model.

increased investment in the first brood has been described before as an adaptive defensive mechanism of *Daphnia* to sterilizing parasites (Ebert, 2005; Hall et al., 2007). The above observations in the three-way ANOVA's of the presence of a clone × carbaryl interaction and the absence of a clone × parasite interaction for the observed life-history endpoints, justifies the choice made regarding the clones to work with.

Overall, we observed strong differences in carbaryl × parasite interactions among the two studied clones for life history endpoints (Two-way ANOVA's, Table 1). First, while no interaction between parasite and carbaryl for survival was found for the less sensitive clone, a synergistic interaction was detected with the sensitive one. Coors et al. (2008) and Coors and De Meester (2008) also found a synergistic interaction between parasite and carbaryl for survival in another *D. magna* clone. Coors et al. (2008) also found a synergistic interaction on sterilization at day 10, but, as noted earlier, interactive effects on this endpoint could not be assessed in the present study because sterilization had already reached 100% in all parasite and combined treatments at day 10. Second, differences in interactions between both clones were also noted for early reproduction: a synergistic effect was detected for the carbaryl-sensitive clone while no significant interaction was detected for the less

**Table 2**
Summary of three-way ANOVA and two-way ANOVA for the two clones for physiological endpoints. Significant *p*-values at the 95% significance level are shown in bold. If significant parasite × carbaryl interactions were detected for a clone, the observed effect in the combined treatment (Eq. (2)), the predicted effect based on the Independent Action model (Eq. (1)) and the interaction type are also provided.

| | df | MS | *F* | *p* | Observed effect $E_{PC}$ | Predicted effect $E_{PC,\ predicted}$ | Interaction type |
|---|---|---|---|---|---|---|---|
| Filtration rate | | | | | | | |
| *Three-way ANOVA* | | | | | | | |
| Clone | 1 | 0.434 | 17.183 | **<0.001** | | | |
| Parasite | 1 | 0.142 | 5.609 | **0.031** | | | |
| Carbaryl | 1 | 0.027 | 1.057 | 0.319 | | | |
| Clone × Parasite | 1 | 0.037 | 1.446 | 0.247 | | | |
| Clone × Carbaryl | 1 | 0.092 | 3.624 | 0.075 | | | |
| Parasite × Carbaryl | 1 | 0.012 | 0.472 | 0.502 | | | |
| Clone × Parasite × Carbaryl | 1 | 0.001 | 0.026 | 0.873 | | | |
| Error | 16 | 0.025 | | | | | |
| *Two-way ANOVA for the two clones* | | | | | | | |
| Parasite × Carbaryl at the level of Clone NS | | | | 0.818 | | | |
| Parasite × Carbaryl at the level of Clone S | | | | 0.055 | | | |
| Energy reserves – Protein | | | | | | | |
| *Three-way ANOVA* | | | | | | | |
| Clone | 1 | 0.172 | 74.320 | **<0.001** | | | |
| Parasite | 1 | 0.027 | 11.471 | **0.004** | | | |
| Carbaryl | 1 | 0.184 | 79.260 | **<0.001** | | | |
| Clone × Parasite | 1 | <0.001 | 0.002 | 0.969 | | | |
| Clone × Carbaryl | 1 | 0.159 | 68.502 | **<0.001** | | | |
| Parasite × Carbaryl | 1 | 0.002 | 1.022 | 0.329 | | | |
| Clone × Parasite × Carbaryl | 1 | 0.005 | 1.951 | 0.184 | | | |
| Error | 14 | 0.002 | | | | | |
| *Two-way ANOVA for the two clones* | | | | | | | |
| Parasite × Carbaryl at the level of Clone NS | | | | 0.645 | | | |
| Parasite × Carbaryl at the level of Clone S | | | | 0.279 | | | |
| Energy reserves – Carbohydrate | | | | | | | |
| *Three-way ANOVA* | | | | | | | |
| Clone | 1 | 0.629 | 96.901 | **<0.001** | | | |
| Parasite | 1 | 0.307 | 47.324 | **<0.001** | | | |
| Carbaryl | 1 | 0.342 | 52.687 | **<0.001** | | | |
| Clone × Parasite | 1 | 0.004 | 0.576 | 0.460 | | | |
| Clone × Carbaryl | 1 | 0.312 | 48.004 | **<0.001** | | | |
| Parasite × Carbaryl | 1 | 0.011 | 1.750 | 0.207 | | | |
| Clone × Parasite × Carbaryl | 1 | 0.010 | 1.583 | 0.229 | | | |
| Error | 14 | 0.006 | | | | | |
| *Two-way ANOVA for the two clones* | | | | | | | |
| Parasite × Carbaryl at the level of Clone NS | | | | 0.963 | | | |
| Parasite × Carbaryl at the level of Clone S | | | | 0.130 | | | |
| Energy reserves – Lipid | | | | | | | |
| *Three-way ANOVA* | | | | | | | |
| Clone | 1 | 0.042 | 4.971 | **0.044** | | | |
| Parasite | 1 | 0.004 | 0.489 | 0.497 | | | |
| Carbaryl | 1 | 0.045 | 5.375 | **0.037** | | | |
| Clone × Parasite | 1 | 0.003 | 0.413 | 0.532 | | | |
| Clone × Carbaryl | 1 | 0.003 | 0.375 | 0.551 | | | |
| Parasite × Carbaryl | 1 | 0.002 | 0.231 | 0.638 | | | |
| Clone × Parasite × Carbaryl | 1 | <0.001 | 0.084 | 0.777 | | | |
| Error | 13 | 0.008 | | | | | |
| *Two-way ANOVA for the two clones* | | | | | | | |
| Parasite × Carbaryl at the level of Clone NS | | | | 0.867 | | | |
| Parasite × Carbaryl at the level of Clone S | | | | 0.672 | | | |
| Pro-phenoloxidase expression | | | | | | | |
| *Three-way ANOVA* | | | | | | | |
| Clone | 1 | 0.002 | 0.007 | 0.934 | | | |
| Parasite | 1 | 0.351 | 1.533 | 0.235 | | | |
| Carbaryl | 1 | 0.758 | 3.308 | 0.089 | | | |
| Clone × Parasite | 1 | 0.037 | 0.162 | 0.693 | | | |
| Clone × Carbaryl | 1 | 0.221 | 0.966 | 0.341 | | | |
| Parasite × Carbaryl | 1 | 0.050 | 0.219 | 0.646 | | | |
| Clone × Parasite × Carbaryl | 1 | 0.094 | 0.411 | 0.531 | | | |
| Error | 15 | 0.229 | | | | | |
| *Two-way ANOVA for the two clones* | | | | | | | |
| Parasite × Carbaryl at the level of Clone NS | | | | **0.035** | −5.21 | −21.79 | antagonism |
| Parasite × Carbaryl at the level of Clone S | | | | 0.917 | | | |

Table 2 (*Continued*)

| | df | MS | *F* | *p* | Observed effect $E_{PC}$ | Predicted effect $E_{PC,\ predicted}$ | Interaction type |
|---|---|---|---|---|---|---|---|
| Electron transfer system [a] | | | | | | | |
| Parasite | 1 | 0.003 | 6.700 | **0.032** | | | |
| Carbaryl | 1 | 0.001 | 3.400 | 0.104 | | | |
| Parasite × Carbaryl | 1 | 0.002 | 10.498 | **0.008** | −0.02 | −0.14 | antagonism |
| Error | 8 | 0.000 | | | | | |
| Acethylcholinesterase activity [a] | | | | | | | |
| Parasite | 1 | 0.001 | 0.119 | 0.738 | | | |
| Carbaryl | 1 | 0.000 | 0.015 | 0.907 | | | |
| Parasite × Carbaryl | 1 | 0.004 | 0.924 | 0.364 | | | |
| Error | 8 | 0.005 | | | | | |

df, degree of freedom; MS, mean square; *F*: *F*-value; *p*: *p*-value.

[a] No three-way ANOVA analysis possible due to lack of data in the clone S combined treatment as a consequence of high mortality. Two-way ANOVA analysis was performed on the level of clone NS instead.

sensitive clone. Third, an antagonistic interaction was observed for body length for the less carbaryl sensitive clone, while a synergistic interaction was detected for the sensitive clone. In summary, only synergistic interactions were found for the carbaryl sensitive clone while either no or anatagonistic interactions were observed for the less sensitive clone. The latter is interesting, as Holmstrup et al. (2010) note in their review that all interactions between pathogens and chemicals reported so far are synergistic and none were antagonistic. The difference with our study might be related to a different exposure scenario: exposures to the stressors in the studies reported in the review by Holmstrup et al. (2010) generally lasted for a longer period than in our study. In our study, exposure to both *P. ramosa* spores and carbaryl ended on day 6, while endpoints were recorded on day 10.

With respect to the measured physiological endpoints, differences in response to carbaryl were detected among the two clones with respect to protein and carbohydrate reserves (Three-way ANOVA's, Table 2), confirming the difference in sensitivity to carbaryl between the two clones. Mobilization and shifts in energy reserves have previously been reported after exposure to different stressors, including pesticides (Calow, 1991; De Coen and Janssen, 2003; Nath et al., 1997). Interestingly, parasite exposure also induced significant shifts in energy reserves in both clones (Fig. 2B and C, Table 2, three-way ANOVA): carbohydrate reserves increased and protein reserves decreased. This shift could be related to the parasite channeling and using different forms of reserves of the *Daphnia* into its own development (Ebert, 2005; Ebert et al., 2004). The strong positive effect of carbaryl in the sensitive clone on ETS reflects an increase in energy demand (Fig. 2E). This suggests an increased investement of energy in mechanisms to cope with the effect of carbaryl on the organism. The two clones also differed in the activity of acetylcholinesterase (AChE) upon carbaryl exposure. Carbaryl acts as a quasi-irreversible inhibitor of AChE. However, in contrast to what would be expected based on this inhibition, an increase in AChE activity was observed for the carbaryl sensitive clone (Fig. 2F). This could be an indirect effect, related to the negative effect carbaryl had on the body length of this clone. This is supported by a recent review by Domingues et al. (2010) which considers size as an important factor affecting AChE, with smaller individuals of species, including *Daphnia magna* and *Daphnia similis*, generally exhibiting higher AChE activity than their larger conspecifics. In addition, Xuereb et al. (2009) reported a strong negative correlation between AChE activity and body weight for the aquatic invertebrate *Gammarus fussarum*. In a similar context, Chandrasekara and Pathiratne (2007) reported body size-related differences in the inhibition of brain AChE activity in juvenile tilapia. Similar to our observations for life-history endpoints, we did not detect any significant clone × parasite interactions for the physiological endpoints with the three-way ANOVA's, indicating that both clones responded in a similar way to *P. ramosa* infection. We detected a significant parasite × carbaryl interaction for proPO expression (an antagonistic one), but only in the less carbaryl-sensitive clone (Table 2, Two-way ANOVA). The latter observation again points to differences in parasite × carbaryl interactions between both clones.

Interactions between genotype and the response to two different environmental stressors have not often been studied yet. Muyssen et al. (2010) studied interactions between temperature and cadmium on both life-history and physiology in three different *D. magna* clones. They detected significant clone × cadmium × temperature interaction effects for two out of three endpoints for which there was a difference in cadmium susceptibility among the three clones, which was reflected by a significant clone × cadmium interaction term. In other words, they too found that stressor interactions (cadmium × temperature) were different among clones that differed in susceptibility to one of these stressors (cadmium).

Different physiological mechanisms may underlie the differences in parasite × carbaryl interactions in life history endpoints between the two clones. Based on the limited available knowledge regarding parasite × chemical interactions, these mechanisms could be related to (i) toxicant-induced reduction in filtration rate resulting indirectly in reduced intestinal exposure to and infection risk by the parasites (Restif and Kaltz, 2006; Auld et al., 2012; Ebert et al., 1996) or (ii) the immuno-modulatory action of chemicals (e.g. Galloway and Depledge, 2001; Galloway and Handy, 2003; Ville et al., 1997). The first mechanism, however, has likely not played a role in our model system, as the absence of a clone × carbaryl interaction for filtration rate (Table 2, three-way ANOVA) indicates no difference among the two clones in how filtration rate is affected by carbaryl. In addition, such a mechanism would likely result in antagonistic interactions in both clones, which is not what we observed here. In contrast, the second mechanism is more plausible. Indeed, different carbaryl × parasite interactive effects between the two clones were observed for proPO expression, i.e. antagonism in the non-sensitive clone and no interaction in the sensitive clone. This is a key enzyme of the immune system response which has been suggested as a useful indicator of immunocompetence in arthropods (Adamo et al., 2001) and which has been shown earlier to be responsive to *P. ramosa* infection (Labbé and Little, 2009, Mucklow et al., 2004). Furthermore, Coors et al. (2008) already argued that their observed synergistic interactions between *P. ramosa* and carbaryl for sterilization and survival (in another *D. magna* clone) could have been the result of such immuno-suppressive activity of carbaryl. All this suggests that differences in the immuno-modulatory activity of carbaryl in both clones may indeed be at the basis of the very different parasite × carbaryl interactive effect between both clones, as observed across the variety of life-history endpoints investigated here.

Collectively, our data show differences in parasite × carbaryl interaction effects among two *Daphnia* clones, with the clone having a higher sensitivity to the chemical stressor exhibiting only synergistic interaction effects and the clone with lower sensitivity to the chemical stressor only exhibiting antagonistic interaction effects. We observed this both for life-history and physiological endpoints. This is in agreement with our hypothesis, derived from the findings of Holmstrup et al. (2010), that sensitive genotypes would be more prone to synergistic effects upon exposure to combined stressors. As our study involved only two clones, however, it does not provide a solid test of this hypothesis, but rather it illustrates that genetically different genotypes (clones) of the same species may strongly differ in their response to mixed stressors and, in our study, do so in a pattern that corresponds to expectations generated by this hypothesis. Further, it should be emphasized that the conclusions drawn in this study are only demonstrated for the particular combination of concentrations or stressor levels of both stressors used. Thus, follow-up studies with multiple clones and a broader range of concentrations or stressor levels would be required to test the broader validity of our hypothesis.

## 5. Conclusions

When analyzing carbaryl × parasite interaction effects between a clone that is highly sensitive and one that is much less sensitive to carbaryl, we observed significant interactions in three out of nine endpoints tested in the sensitive clone and three out of ten endpoints for the less sensitive clone. The interaction effects observed for the less sensitive clone were all antagonistic, while only synergistic interactions were detected in the carbaryl sensitive clone.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2013.01.008.

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