Fitness and virulence of a bacterial endoparasite in an environmentally stressed crustacean host

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SUMMARY

Host-parasite interactions are shaped by the co-evolutionary arms race of parasite virulence, transmission success as well as host resistance and recovery. The virulence and fitness of parasites may depend on host condition, which is mediated, for instance, by host energy constraints. Here, we investigated to what extent stress imposed by predation threat and environmental pollutants influences host-parasite interactions. We challenged the crustacean host *Daphnia magna* with the sterilizing bacterial endoparasite *Pasteuria ramosa* and simultaneously exposed the host to fish kairomones, the pesticide carbaryl or both stressors. While parasite virulence, measured as impact on host mortality and sterilization, increased markedly after short-term pesticide exposure, it was not influenced by predation threat. Parasite fitness, measured in terms of produced transmission stages, decreased both in fish and pesticide treatments. This effect was much stronger under predation threat than carbaryl exposure, and was attributable to reduced somatic growth of the host, presumably resulting in fewer resources for parasite development. While the indirect impact of both stressors on spore loads provides evidence for host condition-dependent parasite fitness, the finding of increased virulence only under carbaryl exposure indicates a stronger physiological impact of the neurotoxic chemical compared with the effect of a non-toxic fish kairomone.

Key words: *Daphnia magna*, *Pasteuria ramosa*, fish predation, carbaryl, insecticide, interaction, toxicity, virulence, parasite transmission, host condition dependence.

INTRODUCTION

In the antagonistic host-parasite relationship, the virulence of the parasite and the defence response of the host lead to a co-evolutionary 'arms race' (Dawkins and Krebs, 1979) in which the parasite often has the advantage of shorter generation times and thus a greater potential for adaptation. The trade-off theory (Anderson and May, 1982) states that parasite virulence, transmission rate and host recovery are linked with each other in a way that results in constraints for the evolution of parasite virulence. This theory has been the subject of debate due to a lack of supporting evidence and limited possibilities to account for all inherent complexities in host-parasite systems (Alizon *et al*. 2009). Actual trade-offs between transmission, virulence and recovery vary widely depending on the host-parasite system studied (Ebert and Bull, 2003). It is part of this complexity that the virulence of parasites can be expected to increase in stressed hosts (i.e., hosts exposed to detrimental environmental conditions), as has been observed in food-limited hosts, while the consequences of enhanced virulence for the fitness of the parasite may vary depending on transmission mode and specific time- and resource thresholds for the production of parasite transmission stages (Bedhomme *et al*. 2004; Jokela *et al*. 2005; Restif and Kaltz, 2006). Trade-offs within the host regarding resource allocation between growth, reproduction and immune defence further complicate the relationship between parasite virulence and host condition. In order to optimize overall host fitness, the resource proportion allocated to immune defence should increase with increasing parasite pathogenicity and strongly decrease below a certain threshold of resource acquisition (Medley, 2002).

From the perspective of the parasite, adaptation should lead to an optimal level of virulence enabling a maximum exploitation of the host and, at the same time, minimize potential disadvantages that would result in reduced transmission rates. Recently, Jensen *et al*. (2006) provided empirical evidence for this 'optimal virulence' model. Using a host-parasite model, in which the bacterial parasite *Pasteuria ramosa* sterilizes the crustacean host *Daphnia magna* in order to monopolize host resources for parasite growth, these authors showed experimentally that the production of parasite transmission stages peaks at intermediate host longevity. Hosts killed earlier by the parasite contained less transmission stages, likely due to a minimum time needed for parasite

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development, while hosts killed later contained less transmission stages, possibly due to slow parasite growth caused by resource limitation.

In general, environmental factors, such as food availability, that influence host condition may significantly alter parasite virulence and, in the long term, shape the co-evolution of host-parasite systems. Using *D. magna* and *P. ramosa*, the same host-parasite model as Jensen *et al*. (2006) and Little *et al*. (2008), we assessed the influence of environmental stress on parasite fitness and virulence. We selected 2 environmental factors: (a) fish predation threat and (b) exposure to a model pesticide, the N-methyl carbamate carbaryl. Planktivorous fish are dominant and common predators of *Daphnia*, driving prey population dynamics and inducing specific anti-predator defences in the prey (Kerfoot and Sih, 1987; Boersma *et al*. 1998). *D. magna* inhabits small eutrophied ponds and shallow lakes, which are often located in areas of intensive agriculture. Hence, contamination with pesticides may be common in such ponds and lakes. Natural *D. magna* populations are therefore likely often exposed to combinations of pollutants and natural antagonists such as predators and parasites. While we reported previously on the enhancing effect of carbaryl exposure on parasite virulence in this host-parasite model (Coors *et al*. 2008), we focus here on the questions (i) whether this enhanced virulence can already be triggered by a short pulse of carbaryl exposure or whether it develops only after long-term exposure of the host, (ii) whether a biotic antagonist can lead to similarly enhanced parasite virulence as exposure to a pollutant, and (iii) whether parasite fitness is impaired or promoted in this host-parasite model when the host is challenged by additional factors.

In the context of the present study, we use the term 'virulence' in a broad sense, referring to the reduction in host fitness attributable to parasitic infection (Alizon *et al*. 2009). We used an increase in host mortality and host sterilization, which both have strong impacts on host fitness, as measures of virulence. As a measure of parasite fitness, we quantified the amount of parasite transmission stages in the host, a convenient measure employed previously in this host-parasite model (Jensen *et al*. 2006; Little *et al*. 2008).

MATERIALS AND METHODS

Host-parasite system

Daphnia magna Straus (Crustacea: Cladocera), being a key grazer of algae and prey to larger invertebrates and fish, is a keystone species of shallow and eutrophied lentic freshwater ecosystems. Under favourable conditions, *D. magna* reproduces parthenogenetically by releasing distinct broods of genetically identical offspring shortly before each moult. The *D. magna* clone (M10) used in the present study was originally isolated as a dormant egg from a sediment core of Oud Heverlee Pond (Belgium), and corresponds to a time at which the pond was characterized by high fish densities (Cousyn *et al*. 2001). The clone has been cultured for several generations as a clonal lineage in the laboratory. Pre-tests had shown that it responds to fish kairomones (e.g., by maturing at a smaller body size) and that it is neither resistant against nor extremely susceptible to *Pasteuria ramosa* infection in comparison with other clones. Stock culturing of *D. magna*, maintenance of fish and experiments were performed in an environmental chamber at 19 °C +/− 2 °C under diffuse light with a light/dark cycle of 18 h/6 h. Artificial freshwater (ADaM, Klüttgen *et al*. 1994) was used as medium, and the green algae *Scenedesmus obliquus* was provided as a food source.

The gram-positive bacterium *P. ramosa* Metchnikoff 1888 is an obligate endoparasite of *Daphnia* and is reported to occur across Europe and North America (Ebert, 2005). *P. ramosa* channels energy resources of the host to the development of new parasite transmission stages by irreversibly sterilizing the host. *P. ramosa* completes its life cycle within its host; transmission stages (infective spores) are only released after the death of the host (Ebert *et al*. 2004). These spores accumulate in the sediment and represent an infection source to *Daphnia*.

Experimental set-up

P. ramosa spores were obtained by exposing *D. magna* of the experimental clone (M10) in the laboratory to sediment from a pond with a history of *P. ramosa* epidemics (Decaestecker *et al*. 2007). A standardized spore solution was produced by grinding the obtained infected *D. magna* and passing it through a 60 μm membrane. Placebo solutions for non-parasite challenged control treatments were prepared in the same way from healthy stock culture daphnids. Repeated observations failed to detect the parasite (*P. ramosa*) in the pond from which the host clone was derived from (Jansen *et al*., unpublished data). The experiment was initiated with 3rd brood neonates (< 24 h) born to females that had been kept under standardized conditions (cultures of single females fed 2×10^5 cells/ml *S. obliquus* daily after reaching maturity, at 19 °C and 16 h/8 h photo-period) for at least 3 generations. The 3 factors parasite challenge, carbaryl exposure and fish predation threat were applied separately or in combination, resulting in a total of 11 different treatments. This design involved 3 full-factorial subsets that shared some of the treatments, while each subset was directed at one of the 3 research questions (Fig. 1). The subsets were: (a) presence/absence of parasites in the absence

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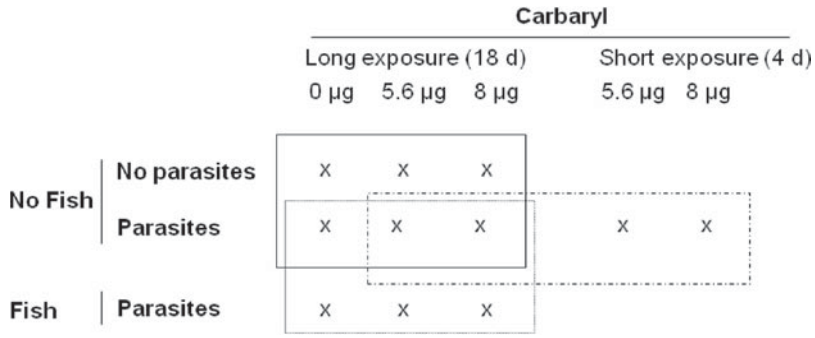

Fig. 1. Design of the experiment with 3 different factors, resulting in 11 different treatment combinations applied to the host *Daphnia magna*: presence or absence of fish kairomones, challenge with spores of the parasite *Pasteuria ramosa* or placebo solution, long-term (18 days) or short-term (first 4 days) exposure to the pesticide carbaryl at 2 different concentration levels (5·6 or 8·0 μg/L) or solvent control treatment. The 3 boxes indicate the different sets of treatments analysed together in one analysis of variance or covariance. Subset (a), solid line: parasite and carbaryl as main factors; subset (b), dashed line: carbaryl exposure duration and concentration level as main factors; subset (c), dotted line: parasite and fish as main factors.

of fish combined with long-term exposure to carbaryl at 2 concentrations and a solvent control; (b) short-term and long-term exposure to carbaryl at 2 concentrations in the presence of parasites and the absence of fish; and (c) presence/absence of fish threat combined with parasite challenge and long-term exposure to carbaryl at 2 concentrations and a solvent control. Each treatment was replicated 5 times and each replicate contained 13–16 (mostly 15) *D. magna*, kept together as an experimental group throughout the experiment. During the first 4 days of the experiment, each experimental unit represented a volume of 20 ml, which was increased to 500 ml for the remainder of the experiment (days 4 to 18). Daphnids were fed daily with *S. obliquus* and were transferred to freshly prepared medium every second day. The food level increased from $1{\cdot}0\times10^5$ cells/ml (0·75 mg C/L) daily on days 0 to 3 to daily $1{\cdot}2\times10^5$ cells/ml (0·90 mg C/L) on days 4 to 10 and finally to 2×10^5 cells/ml (1·5 mg C/L) daily from day 11 onwards.

Parasite challenge was applied by adding 1 ml of a solution containing 10^6 mature *P. ramosa* spores to 19 ml of medium on days 0 and 2, while parasite-free treatments received 1 ml of placebo solution. Challenge by *P. ramosa* spores ceased at day 4, when daphnids were transferred to the larger vessels containing 500 ml of medium.

Carbaryl (1-naphthyl methylcarbamate, CAS 63-25-2, purity 99·8%, Sigma-Aldrich, Germany) stock solutions were prepared in ethanol and stored in brown glass vessels at –20 °C. Exposure medium containing carbaryl was prepared freshly from these stock solutions every second day when the medium of all experimental vessels was renewed. The final solvent concentration was the same in all treatments (0·05 ml/L). Carbaryl was applied at 2 concentration levels (5·6 and 8·0 μg/L) that were found in pilot experiments to cause no acute toxic effects in absence of other stressors (A. Coors, unpublished data). Carbaryl exposure was applied either only during the first 4 days (short-term exposure) or during the whole experimental period of 18 days (long-term exposure).

Daphnia recognizes the presence of fish by sensing chemicals that are released from the predator (so-called kairomones). Adaptive behavioural and life-history responses of *Daphnia* to fish predation threat can therefore be induced by exposing the prey to fish-conditioned medium (Boersma *et al*. 1998). We obtained medium conditioned with fish kairomones by keeping 3 fish (*Leuciscus idus*) in an aquarium with 20 L of ADaM for 24 h, filtering the medium (0·45 μm), and diluting it to an equivalent of 3 fish per 80 L. Fish were fed daily outside the aquarium with live *D. magna* from stock culture and conventional fish food.

During the course of the experiment, survival of *D. magna* was checked daily and occurrence and time to sterilization were recorded for each surviving individual. At the end of the experiment, daphnids from parasite challenge treatments were frozen individually and stored at −20 °C. We determined the number of mature *P. ramosa* spores in 5 randomly selected infected daphnids from each experimental unit by grinding each individual in 300 μl of distilled water and counting spores at 400× magnification in a Bürker chamber. In 2–4 replicates of each treatment, the length of at least 5 individual daphnids per replicate was measured at the end of the experiment. This resulted in a total of 120 infected host individuals for which data on both body length and spore load were available.

Statistical analysis

For the endpoint mortality rate per vessel (at day 4 and day 18) and sterilization rate per vessel (excluding dead *Daphnia*), we conducted analyses of variance

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on 3 subsets of the 11 treatments as indicated in Fig. 1. We performed two-way ANOVAs to test for effects of long-term carbaryl exposure and parasite challenge in the absence of fish threat (a subset of the results was reported earlier by Coors *et al*. 2008). We further conducted two-way ANOVAs to test for the effect of carbaryl exposure duration and carbaryl concentration level on parasite-challenged hosts in the absence of fish threat, and two-way ANOVAs to test for effects of fish threat and long-term carbaryl exposure on parasite-challenged hosts. The latter 2 ANOVAs (within parasite-challenged hosts) were also performed for the 2 variables parasite spore load and host body length (both averaged per vessel). Because the 3 subsets of ANOVA all included the 2 treatments with parasite challenge/fish absence/long-term carbaryl exposure, we corrected for multiple testing by the Bonferroni method and used an actual alpha value of 0·05/3 = 0·0166. Similar to conducting an ANCOVA as a follow-up analysis of a multivariate analysis of variance (recommended, for example, by Harlow (2005) and Tabachnik and Fidell, 2007), we additionally analysed spore load and host body length using 1 of the 2 variables as a covariate, in order to test if effects on the first variable were still apparent when effects on the second variable were taken into account. In all analyses, mortality and sterilization rates as well as host body length and spore loads complied with the assumptions of normality and homogenous variance, judged by visual inspection of residuals and Bartlett's test. Data were thus not transformed prior to analysis. Statistical analysis was performed using Statistica (v.8.0, StatSoft Inc., 2007).

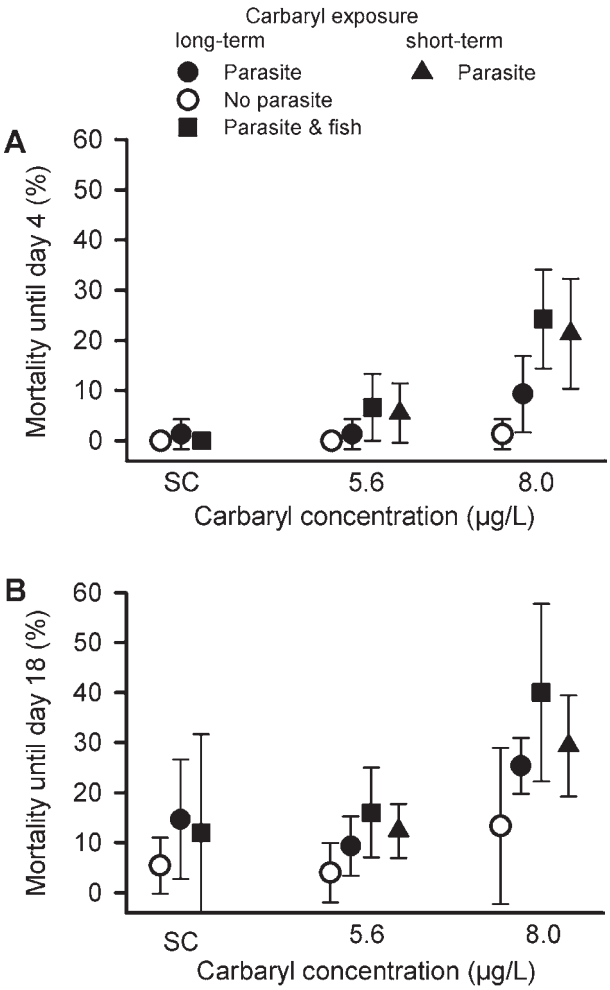

Fig. 2. Mortality of the host *Daphnia magna* until day 4 (A) and until day 18 (B). Neonate hosts were challenged by spores of the parasite *Pasteuria ramosa* or by a placebo solution and exposed to the pesticide carbaryl in absence or presence of fish kairomones. Carbaryl exposure was either short-term (4 days) or long-term (18 days). SC, carbaryl-free solvent control. Given are means and standard deviation for $n = 5$ replicates per treatment.

RESULTS

Host mortality

The lethality of either *P. ramosa* challenge or carbaryl exposure alone was very low, with almost 100% of *D. magna* surviving until day 4 and more than 80% until the end of the experiment at day 18. Yet, higher mortality was observed in parasite-challenged hosts exposed to 8·0 µg/L carbaryl both at day 4 and at day 18. (Fig. 2). When analysing subset (a), the interaction between parasite challenge and long-term carbaryl exposure was not significant at day 18, but the main factors were significantly affecting survival (Table 1). At day 4, the interaction between parasite challenge and carbaryl exposure was also not significant when Bonferroni correction for multiple testing was considered. Short-term and long-term carbaryl exposure treatments were pooled to assess these effects on mortality until day 4, because different exposure conditions were applied only from day 4 onwards. The absence of a difference in mortality until day 4 among short- and long-term exposure treatments was confirmed by comparing the treatments with the same carbaryl exposure concentration using *t*-tests (both $P > 0{\cdot}05$).

The analysis of subset (b) showed that there was no detectable effect of carbaryl exposure duration on the mortality rate of parasite-challenged hosts until the end of the experiment, while the carbaryl concentration level significantly affected mortality until day 18 independently from exposure duration (Table 1).

The analysis of the third ANOVA subset (subset (c), Fig. 1), which involved the long-term exposure of parasite-challenged *D. magna* to the presence or absence of fish kairomones in combination with carbaryl, revealed no significant interaction between the factors on mortality rates at day 18 (Table 1). Fish threat, as a main factor, did not affect survival of parasite-challenged hosts until day 18, while the exposure to carbaryl had a significant impact.

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Table 1. Results of the two-way ANOVAs with regard to host mortality for the 3 different data subsets outlined in Fig. 1

(The investigated factors were parasite (*Daphnia magna* challenged by *Pasteuria ramosa* or a placebo solution), carbaryl (solvent control, 5·6 and 8·0 μg/L carbaryl), carbaryl concentration (5·6 and 8·0 μg/L), duration of carbaryl exposure (4 days or 18 days), and fish (presence or absence of fish kairomones). Subsets (b) and (c) involved only parasite-challenged hosts.)

Data subset and parameter	D.F.	MS	F	*P*
Subset (a) – Mortality until day 4				
parasite	1	352·0	8·79	0·006
carbaryl	2	215·1	5·37	0·009
parasite*carbaryl	2	142·6	3·56	0·039
error	34	40·1		
Subset (a) – Mortality until day 18				
parasite	1	588·4	6·78	0·016
carbaryl	2	430·2	4·96	0·016
parasite*carbaryl	2	28·1	0·32	0·727
error	24	86·7		
Subset (b) – Mortality until day 18				
carbaryl concentration	1	1356·1	27·37	0·0001
exposure duration	1	62·3	1·26	0·279
concentration*duration	1	1·1	0·02	0·883
error	16	49·5		
Subset (c) – Mortality until day 18				
fish	1	290·2	1·76	0·197
carbaryl	2	1290·0	7·81	0·002
fish*carbaryl	2	188·0	1·14	0·337
error	24	165·1		

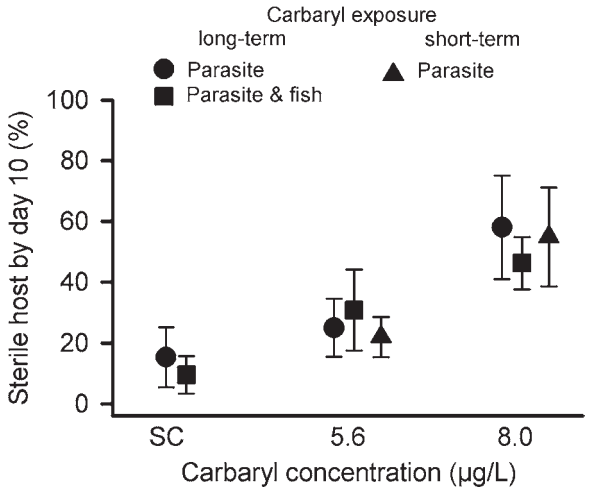

Fig. 3. Parasite-induced sterilization of the host *Daphnia magna* at day 10 (before first reproduction of the host). In addition to the challenge by spores of the parasite *Pasteuria ramosa*, neonate hosts were exposed to fish kairomones and the pesticide carbaryl (either for the full 18 days or a short period of the first 4 days only). Placebo treatments are not shown as no infections occurred. SC, carbaryl-free solvent control. Given are means and standard deviation for $n = 5$ replicates per treatment.

Parasite-induced host sterilization

From the *D. magna* exposed to parasite spores, 100% were sterilized at the end of the experiment for all treatments that involved carbaryl exposure, and 98·7% were sterilized in the 2 solvent-control treatments (with and without fish kairomones). Given these sterilization rates at day 18, the percentage of sterilized hosts at time-points earlier in the experiment reflects the speed with which the parasite sterilized the host. At day 10, all experimental daphnids were either carrying the first clutch of offspring in their brood pouch or were irreversibly sterilized before any reproduction. Hence, sterilization rates at day 10 are used as an additional measure of parasite virulence (Fig. 3), because particularly fast sterilization has a strong impact on host fitness.

The analysis of subset (c) showed that carbaryl exposure significantly enhanced sterilization rates in parasite-challenged hosts at day 10, whereas exposure to fish kairomones had no effect (Table 2). The interaction term between fish and carbaryl was insignificant. Neither fish threat nor carbaryl exposure induced sterilization of *Daphnia* in the absence of parasite-challenge. Thus, we found an interaction of parasite infection with carbaryl exposure but not with fish threat.

There was no difference in the effect of carbaryl on host sterilization rates between long-term and short-term exposure (subset (b), Table 2), while carbaryl concentration level affected the sterilization rate independently from the exposure duration. Hence, carbaryl exposure during the first 4 days only was sufficient to result in enhanced host sterilization speed, manifested several days after the carbaryl exposure stopped.

Production of parasite transmission stages

The presence of fish kairomones affected the average amount of *P. ramosa* endospores per infected host (spore load) independently of carbaryl exposure, while the effect of carbaryl was marginally non-significant (subset (c), Table 3). In the solvent controls, the spore load was, on average, 2·2 million endospores per host in the absence of fish kairomones compared with 1·5 million endospores in the presence of fish kairomones. Partial eta-squared (partial η^2) as effect size measure indicated a medium-sized effect of fish (partial $\eta^2 = 0{\cdot}42$), but a rather small effect attributable to carbaryl (partial $\eta^2 = 0{\cdot}21$). Despite the absence of a significant overall carbaryl effect in the ANOVA of subset (c), the carbaryl concentration level significantly influenced spore loads in the analysis of subset (b) (Table 3). Hosts only shortly exposed to the pesticide tended to

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Table 2. Results of the two-way ANOVAs with regard to speed of host sterilization for the subsets (b) and (c) outlined in Fig. 1

(The investigated factors were carbaryl (solvent control, 5·6 and 8·0 μg/L carbaryl), carbaryl concentration (5·6 and 8·0 μg/L), duration of carbaryl exposure (4 days or 18 days), and fish (presence or absence of fish kairomones). Only treatments with *Pasteuria ramosa* challenge are considered because placebo-treated *Daphnia magna* were not sterilized.)

Data set	D.F.	MS	F	*P*
Subset (b)				
carbaryl concentration	1	5465·2	31·5	<0·0001
exposure duration	1	48·1	0·278	0·606
concentration*duration	1	0·03	0·0001	0·990
error	16	173·2		
Subset (c)				
fish	1	115·3	0·90	0·353
carbaryl	2	4021·8	31·3	<0·0001
fish*carbaryl	2	202·2	1·58	0·228
error	24	128·4		

Table 3. Results of the two-way ANOVAs with regard to parasite spore loads at day 18 for the subsets (b) and (c) outlined in Fig. 1

(The investigated factors were carbaryl (solvent control, 5·6 and 8·0 μg/L carbaryl), carbaryl concentration (5·6 and 8·0 μg/L), duration of carbaryl exposure (4 days or 18 days), and fish (presence or absence of fish kairomones) in *Daphnia magna* infected with *Pasteuria ramosa*.)

Data set	D.F.	MS (*10^{11})	F	*P*
Subset (b)				
carbaryl concentration	1	16·1	9·19	0·008
exposure duration	1	7·7	4·39	0·052
concentration*duration	1	1·5	0·88	0·362
error	16	1·8		
Subset (c)				
fish	1	20·2	17·29	0·0004
carbaryl	2	3·7	3·21	0·058
fish*carbaryl	2	0·7	0·56	0·576
error	24	1·2		

contain more parasite spores than hosts exposed over the entire experimental period, but this trend was not significant. The interaction between carbaryl concentration level and exposure duration time was also not significant. Effect sizes (partial η^2) were 0·36 and 0·21 for carbaryl concentration and exposure duration, respectively.

In the smaller subset of experimental units for which data on mean host body length were available (n=2–4 replicates per treatment), ANOVA revealed significant effects of fish kairomones and carbaryl exposure on the body length of parasite-infected hosts (subset (c), Table 4), with the effect of fish being strong (partial η^2 of 0·79) and that of carbaryl medium-sized (partial η^2 of 0·50). Neither carbaryl concentration level nor exposure duration was significant with regard to host body length after Bonferroni correction in the analysis of subset (b). Yet, partial η^2 indicated medium-sized effects for both carbaryl concentration (0·33) and carbaryl exposure duration (0·45). In comparison to the parasite-only treatment, *D. magna* were smaller in both the fish and the highest carbaryl treatment, with hosts only shortly exposed to carbaryl tending to be larger at day 18 than those exposed for the whole experimental period.

The body length of infected hosts and the amount of parasitic spores per host were significantly correlated across all treatments (Pearson r=0·46, P<0·0001, n=120 individual hosts measured; Fig. 4). Two hosts with extremely small body size (both exposed to 8·0 μg/L carbaryl and fish kairomones) were identified as outliers (Grubbs test, P<0·001), but excluding them did not change the observed correlation (Pearson r=0·41, P<0·0001, n=118). Both individuals were also extremes with

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Table 4. Results of the two-way ANOVA for host length and the ANCOVA for spore load, considering host length as covariate, in subsets (b) and (c)

(The investigated factors were carbaryl (solvent control, 5·6 and 8·0 μg/L carbaryl), carbaryl concentration (5·6 and 8·0 μg/L), duration of carbaryl exposure (4 days or 18 days), and fish (presence or absence of fish kairomones) in *Daphnia magna* infected with *Pasteuria ramosa*.)

Data set	D.F.	MS	F	*P*
Subset (b) – Host length				
carbaryl concentration	1	0·0214	4·55	0·061
exposure duration	1	0·0352	17·48	0·023
concentration*duration	1	0·0002	0·04	0·834
error	9	0·0047		
Subset (b) – Spore load				
host length	1	2·2	2·47	0·155
carbaryl concentration	1	1·2	1·40	0·270
exposure duration	1	0·05	0·05	0·822
concentration*duration	1	4·6	5·13	0·053
error	8	0·9		
Subset (c) – Host length				
fish	1	0·2834	45·79	<0·0001
carbaryl	2	0·0369	5·96	0·016
fish*carbaryl	2	0·0135	2·18	0·156
error	12	0·0062		
Subset (c) – Spore load				
host length	1	0·002	0·03	0·865
fish	1	1·96	2·73	0·127
carbaryl	2	1·05	1·46	0·274
fish*carbaryl	2	0·87	1·21	0·335
error	11	0·72		

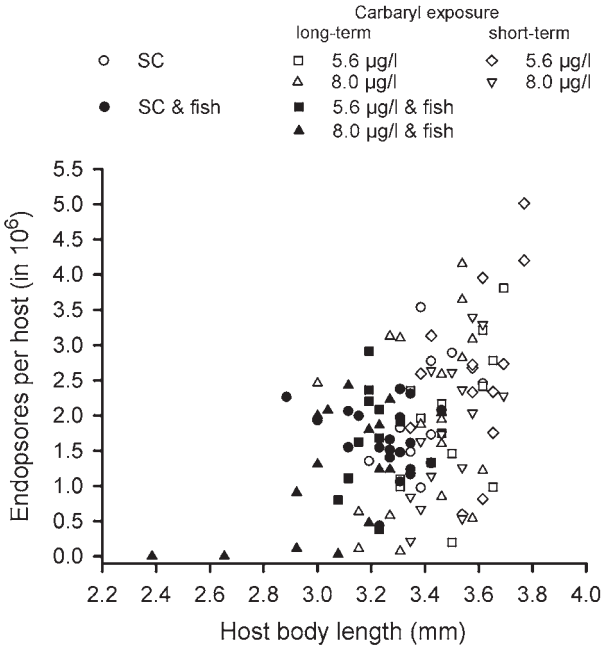

Fig. 4. Association between body length and parasite spore load in the host *Daphnia magna* infected by *Pasteuria ramosa* after exposure to fish kairomones and carbaryl (either for the full 18-day experiment or a short period of the first 4 days only). SC, carbaryl-free solvent control. Given are observations for individual hosts (n=120).

regard to spore load, as no endospores were detected during routine counting in the Bürker chamber and infection could only be confirmed after intensive screening of settled host tissue.

When host body length was taken into account as a covariate in an ANCOVA, neither fish kairomones nor carbaryl exposure had a significant effect on spore loads (subset (c), Table 4), while an ANOVA on the same smaller subset of experimental units produced similar results as reported above for the complete data set (results not shown). Carbaryl exposure duration and concentration level also exhibited no significant effect on spore loads when host body length was used as covariate in ANCOVA (subset (b), Table 4). Hence, effects on host body length and correlation of spore load with host size appeared to fully explain the effects of fish kairomone and carbaryl exposure on parasite spore loads. This finding was further substantiated by the fact that partial η^2 for all main factors were below 0·24 in the ANCOVAs. Fig. 5 illustrates this finding by showing mean spore loads adjusted for the effect of host body size.

DISCUSSION

In the presence of the pesticide carbaryl, the parasite *P. ramosa* exhibited an enhanced virulence in the host

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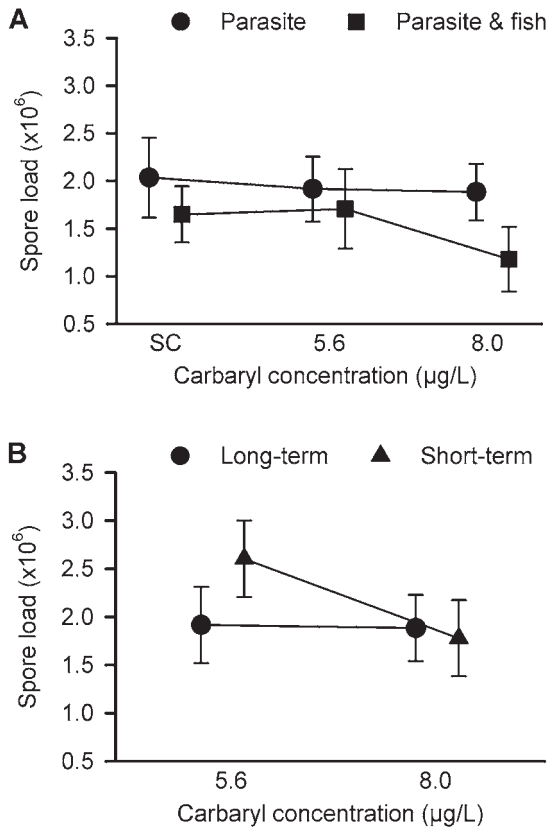

Fig. 5. Interaction plots of ANCOVAs for spore loads in the host *Daphnia magna* infected by *Pasteuria ramosa* using exposure to fish kairomones and carbaryl as main factors (A) or carbaryl exposure duration (either for the full 18-day experiment or a short period of the first 4 days only) and carbaryl concentration level as main factors (B). SC, carbaryl-free solvent control. Given are least square means and their 95% confidence intervals after adjusting for host body length.

D. magna (clone M10), both in terms of increased lethality and accelerated host sterilization. These effects might be due to an immunomodulatory effect of carbaryl, as reported and discussed previously in more detail (Coors *et al*. 2008). The present study shows that a short-term exposure to carbaryl, only during the first 4 days of initial parasite challenge, is already sufficient to lead to an enhanced virulence. This observation strengthens the environmental relevance of increased parasite virulence upon carbaryl exposure. Short exposure periods are typical for pesticide contamination of aquatic environments, because the usage of pesticides in agricultural fields leads to pulsed contamination events, resulting from run-off or spray drift, whereas constant contamination is less likely (Schulz, 2004). This holds true even more for pesticides of low persistence, such as carbaryl, that is quickly hydrolysed at ambient pH (EPA, 2003). As the increased host mortality induced by carbaryl exposure in this host-parasite system is also likely to adversely impact overall parasite fitness (i.e., by reducing the longevity of hosts to less than the time needed for completion of the parasite life cycle; Jensen *et al*. 2006), the high level of virulence in the presence of contaminants can be regarded as disadvantageous for the parasite. In contrast to carbaryl exposure, predation threat, as imposed by the presence of fish kairomones, did not lead to increased virulence, neither in terms of sterilization speed nor host mortality. A previously reported study (Coors and De Meester, 2008) also found no enhanced virulence of *P. ramosa* in the same *D. magna* host clone threatened by an invertebrate predator, the phantom midge *Chaoborus crystallinus*.

Assuming that *P. ramosa* virulence is host condition-dependent, the difference in the effect of the biotic and chemical stressors on virulence observed here likely reflects the much greater impact of the neurotoxic pesticide compared with that of the non-toxic kairomones on host physiology. A toxic chemical disrupts the physiology and thereby results in adverse effects on host condition. Mediated presumably by gene regulation, the effects of kairomones, on the other hand, lead only to a different, but fully functional host physiological state in adapted prey. Such an adaptation can be expected for the *D. magna* clone used here, because it originates from an environment under a high pressure of fish predation. Yet, it remains unclear to what extent evolutionary adaptation of *Daphnia* to fish predation pressure might contribute to the lack of change in parasite virulence observed herein. Further experiments using host clones that show no adaptive response to fish presence would be needed to clarify this aspect.

Examples supporting host condition-dependent virulence are provided by Restif and Kaltz (2006), who reported higher virulence of a bacterial parasite in a ciliate host at a reduced food level, and by Jokela *et al*. (2005), who observed enhanced virulence in a bivalve-trematode host-parasite model related to oxygen depletion and host starvation. In a mosquito-microsporidian host-parasite model, Bedhomme *et al*. (2004) reported not only an increased parasite virulence with decreasing host food levels, but also showed that the fitness of the parasite, as measured by the production of transmission stages, was positively correlated with food availability to the host. The production of *P. ramosa* spores depends highly on exploitable host resources and has been reported previously to be positively correlated with host food level (Carius *et al*. 2001; Ebert *et al*. 2004).

The presence of fish kairomones reduced spore loads and thus adversely affected parasite fitness in the *D. magna* clone investigated. Although the effect of carbaryl on spore loads was far less strong than that of kairomones, there was a trend for a decreased number of parasite spores per host at high and constant exposure to the pesticide carbaryl. Both carbaryl and kairomone exposure lead to reduced

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host body sizes, with the effects on host size being much stronger than those on spore loads. The reduced spore loads could be fully attributed to decreased somatic growth of the host. The underlying reason is likely to be that smaller hosts provide less physical space and fewer energy resources for the development of transmissible stages. Hence, the transmission stage production and thereby the fitness of the parasite was indirectly affected by effects of fish predation threat and pesticide exposure on host somatic growth.

In the case of the pesticide, sublethal exposure may have reduced *Daphnia* filtration rate (as is known for various toxicants; McWilliam and Baird, 2002) and thus lowered food uptake. Additionally, biochemical detoxification of carbaryl may have caused a higher energetic demand in the host. A less pronounced reduction of host body size upon short-term carbaryl exposure in comparison with long-term exposure substantiates transient carbaryl effects on host growth as the underlying reason. A direct inhibitory effect of carbaryl on parasite growth and development might have played an additional role, because carbaryl as well as its main metabolite, 1-naphthol, are known to be toxic to various bacteria (DeLorenzo *et al*. 2001). However, a direct toxic impact on the parasite would only explain reduced parasite fitness, but not the observed increase in parasite virulence or the reduced host body length. The role of direct effects of contaminants on the parasite can hardly be verified experimentally, because *P. ramosa* (an obligate endoparasite) cannot be cultured independently from its host.

The smaller size of kairomone-exposed hosts results from the exhibited anti-predator strategy, which consists of a re-allocation of resources to early reproduction instead of somatic growth. In most of 16 studied *D. magna* clones, Boersma *et al*. (1998) found that reduction in size at maturity was part of the otherwise genetically highly variable response to fish kairomones. This response is advantageous for the prey under size-selective fish predation pressure, and is shown here to result at the same time in a fitness disadvantage for the parasite *P. ramosa*. Predation by fish has been pointed out earlier as a potential factor limiting the spread and prevalence of various parasites in *Daphnia* populations (Duffy *et al*. 2005; Pulkkinen and Ebert, 2006), because parasites may not be able to complete their life cycle within the host before they are consumed by fish together with the host. Yet, these disadvantages regarding parasite fitness do not prevent the co-occurrence of *P. ramosa, D. magna* and planktivorous fish in the field, which has for example been reported for the pond sampled to obtain *P. ramosa* spores in the present study (Decaestecker *et al*. 2002). One factor that enables co-existence of parasite, host/prey and predator may be the typical predator avoidance behaviour of the prey, migration to sediment mediated by negative phototaxis, creating an increased infection risk due to the contact with the sediment spore bank (Decaestecker *et al*. 2002). The absence of increased virulence in the predator-threatened host, observed in the present study, may be another factor enabling this co-existence. While Decaestecker *et al*. (2002) showed a synergistic effect of parasite and predator exposure with respect to infection risk, we show here that there is no synergistic interaction with respect to virulence. In a different *Daphnia*-microparasite system, Lass and Bittner (2002) likewise found no synergistic interaction between the effects of the parasite and predation threat on life-history traits of the host. Following the trade-off theory (Anderson and May, 1982), the level of *P. ramosa* virulence can therefore be interpreted as optimized with regard to the stress level of the host under predation pressure. Taking together the absence of increased parasite virulence and the occurrence of decreased parasite fitness, it appears that the host response to fish predation threat does not imply immediate costs in terms of reduced parasite resistance, at least not in the investigated host (clone)-parasite model. Hence, adaptation to predation threat can impose costs on the prey leading to reduced immune competence (Rigby and Jokela, 2000), but such a trade-off may not be realized under all conditions or in all parasite-host-predator systems.

As the results reported here were obtained with a single host genotype, an investigation of a large number of different host clones would be needed to confirm whether the findings are representative of the species. In *D. magna*, the known genetic variability in its responsiveness to fish predation threat (Boersma *et al*. 1998) as well as in its susceptibility to acute toxicity of carbaryl (Coors *et al*. 2009) and to *P. ramosa* infection (Ebert, 2005; Decaestecker *et al*. 2007) are expected to result in a extensive genetic diversity in the interaction effects of these factors. In the present study, we provide additional evidence for reduced parasite fitness in environmentally stressed hosts. We also demonstrate that, in addition to environmental factors, such as food availability and temperature, exposure to sublethal concentrations of a pesticide has to be considered as a relevant factor in the context of host condition-dependent virulence, parasite fitness and, thus, ecological and evolutionary dynamics of host-parasite interactions in the field.

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