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# Evolutionary ecotoxicology of pesticide resistance: a case study in *Daphnia*

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**Abstract** Natural populations that are exposed to pesticides in their environment may at the same time be exposed to natural stressors like parasites and predators, which may interact with pesticide exposure. This may not only impact target pest species but also a wide variety of non-target species. This review reports on a joint research program in the water flea *Daphnia magna*, a non-target species often used as model organism in ecology and ecotoxicology. The focus is on different aspects that are of key importance to understand the evolutionary ecology of pesticide exposure: (1) the capacity of natural populations to genetically adapt to pesticide exposure (2) the added complexity of synergistic effects caused by simultaneous exposure to natural stressors, and (3) the potential interference of evolutionary costs of adaptation to pesticide exposure. Our results showed that natural populations were able to rapidly evolve resistance to the pesticide carbaryl but at the expense of fitness costs. Individuals selected for carbaryl resistance had higher survival rates when exposed to the pesticide but also a greater susceptibility to the challenge imposed by the bacterial endoparasite *Pasteuria ramosa*. The evolved resistance to carbaryl was in some cases only expressed in the absence of fish kairomones. Further, it became clear that the responses to both exposure to single and combined stressors was for several life history variables strongly dependent upon past exposure to carbaryl. This indicates that past exposures to pesticides are important and can not be neglected when evaluating responses to current stressors.

**Keywords** *D. magna* · Pesticide resistance · Multiple stressors · Genetic adaptation · Evolutionary cost · Synergistic effects

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## Introduction

Anthropogenic stressors exert strong pressure on ecosystems worldwide (Vitousek et al. 1997; Palumbi 2001; King et al. 2010). Chemicals are released into the environment, often leading to changing population dynamics, community composition and ecosystem functioning (e.g. Relyea 2003; Relyea and Hoverman 2008; Geiger et al. 2010). Among these, pesticides are a particular class of chemicals that are used worldwide in agriculture (Palumbi 2001), with increasing concern for biodiversity (Vitousek et al. 1997). This review focuses on two independent research lines in pesticide research: (1) on genetic adaptation and its evolutionary costs, and (2) on interactions of pesticides with other stressors. These two aspects are of key importance to understand the evolutionary ecology of pesticide exposure. The here proposed study on the water flea *Daphnia magna* illustrates an integrated research program including both research lines.

### Genetic adaptation (resistance) and its evolutionary cost

Pesticides are specifically designed to negatively affect populations of pest species. This makes them strong selective factors. As a result, genetic adaptation may occur

in natural populations exposed to pesticides (Carriere et al. 1994; Cothran et al. 2010). Resistance to pesticides can evolve fast (Lopes et al. 2008; Brausch and Smith 2009), and development of pesticide resistance in target species is an important theme in applied evolutionary biology because of the risk of losing mechanisms to control pest species (Raymond et al. 2001). Due to for example run off or aerial transportation (drift or evaporation), pesticides can also reach and negatively affect non-target organisms (Giddings et al. 1996; Davidson 2004; Rohr and Crumrine 2005), and only the least sensitive individuals may survive in contaminated environments (Klerks and Levinton 1989). Pesticide resistance in individuals and populations of non-target species is important as it may determine which non-pest species prosper in areas with intensive agriculture, and as such may modulate how pesticides affect genetic diversity and species diversity.

Besides documenting resistance, evaluating the costs of resistance is also very important, as resistance costs are typically believed to delay resistance evolution (Tabashnik et al. 2005). In addition, costs of resistance may also indirectly impact the fitness of populations and shape interactions with other species in the community. Genetic adaptation to pesticides is expected to be not free of costs (e.g. Duron et al. 2006). As is typical for local adaptation, individuals adapted to a specific stress environment may be expected to be less fit in an environment without the stressor to which they are adapted than individuals not adapted to this stressor (Futuyma 1986; Lenormand et al. 1999). Two main mechanisms have been proposed to explain why increased resistance may involve a metabolic cost under stress-free conditions. First, increased energy allocation to detoxification mechanisms in adapted individuals may explain a decrease in fitness of those individuals in a stressor-free environment, at least to the extent that this increased allocation is constitutively expressed also in the absence of the stressor. Secondly, reduced genetic variability within the adapted population may lead to an inability of a population to adapt to other stressors in the future (Koehn and Bayne 1989; Roff 1997; Athrey et al. 2007).

When individuals exhibit inducible defences against stress, costs can be reduced if the defence is only expressed when the stressor is present. A first process is the induction of protective responses. For instance, organic pollutants often induce enzymes of the monooxygenase system that increase the production of water-soluble metabolites and low toxicity conjugates that can be excreted (Walker et al. 2006), the presence of proteins protecting cells from oxidative stress like glutathione or the induction of stress proteins, molecular chaperones involved in the appropriate folding of proteins to repair the damage caused by the pollutant. A second possible inducible process influences the internal bioavailability of pollutants by binding them to other molecules. Other possibilities are switching on/off of apoptosis genes or genes involved in cell turnover (Walker et al. 2006).

Several studies have documented fitness costs of pesticide exposure (e.g. decrease in the number of juveniles, delay in maturation time) following laboratory experiments in which populations exposed to different concentrations of pollutants in nature were compared (e.g. Carriere et al. 1994). These costs also explain why populations no longer exposed to a certain pesticide tend to lose their resistance. For example, Brausch and Smith (2009) showed that *Daphnia magna* no longer exposed to naphthalene and cyfluthrin lost their resistance to these pollutants. The time to return to the initial susceptibility to these pesticides was longer than the time needed to develop resistance against them, indicating that the kinetics of switching genes on and off are different to the time scales of excretion and uptake and likely reflect differences in selection pressures. A number of other studies did not detect fitness costs of resistance (e.g. Brausch and Smith 2009; Lopes et al. 2008). This seems to suggest that fitness costs are not generally present after selection or that in some studies not all relevant fitness components were measured. Some studies even documented fitness advantages in the absence of pollutants after selection for resistance to pollutants (e.g. Haubruge and Arnaud 2001; Arnaud et al. 2005). Whether resistance evolution carries costs or not may at least partly depend on the molecular mechanism of the evolved resistance.

## Interactions with other stressors

In agricultural areas, organisms are typically not only exposed to pesticide stress but at the same time confronted with natural stressors, both abiotic (e.g. changes in temperature, humidity or acidity) and biotic (parasites, fish predation, competition) (Sih et al. 2004). There is increasing interest in the combined effects of such stressors because some studies reported non-additive effects, including synergistic interactions through which the negative effects of pesticides were magnified in the presence of other stressors (e.g. Relyea and Mills 2001). Pesticides may increase the sensitivity of exposed individuals to natural, biotic stressors altering host-parasite (Coors et al. 2008; Coors and De Meester 2008, 2010; Griggs and Belden 2008; Rohr et al. 2008) or predator-prey interactions (e.g. Hanazato 1995; Relyea and Mills 2001; Pestana et al. 2010). Neither single stressor empirical studies nor models are currently able to predict synergistic interactions, which are of the highest concern.

Interactions with other stressors may underlie "hidden" costs of apparently cost-free resistance (Depledge 1994). One of these hidden costs associated with pesticide resistance may be increased susceptibility to parasites (e.g. Agnew et al. 2004; Duron et al. 2006; Farenhorst et al. 2009). Parasites are an important selection agent in natural

populations. Increased susceptibility to parasites in pollutant-resistant mosquito strains has been documented in several studies (e.g. Berticat et al. 2002; Agnew et al. 2004). Agnew et al. (2004) observed a context-dependent cost associated with alleles that confer resistance to organophosphate insecticides: resistant strains of *Culex pipiens* differ in their cost of resistance relative to a sensitive strain depending on which trait is studied and depending on the presence of the parasite *Vavraia culicis*. Another hidden cost of pesticide resistance may be increased susceptibility to predators. Berticat et al. (2004) showed an increased probability of being consumed by a predator in *C. pipiens* strains carrying a resistance gene, coding an overproduced detoxifying esterase (locus Ester) or an insensitive target gene (Locus ace 1) compared to pesticide susceptible strains.

Most studies either focus on local genetic adaptation and costs of evolution or on direct interactions between stressors. A joint research program including both research themes may provide insight into questions like whether the resistance itself but also its evolutionary costs depend upon combined exposure to other stressors, and whether direct interactions between pesticides and other stressors differ in function of evolved pesticide resistance. The remainder of this review reports on an integrated research program in the water flea *Daphnia magna*, a non-target species often used as a model system in ecotoxicology (Walker et al. 2006). *D. magna* is also a model species in evolutionary ecology (e.g. Cousyn et al. 2001; Decaestecker et al. 2007) and a keystone species in ecosystems, where it occupies an important intermediate trophic position (Lampert 1987). This makes *Daphnia* a particularly useful species for an integrated study of genetic adaptation, evolutionary costs of resistance and interactions between pesticides and biotic stressors. There are a number of studies that document patterns of local genetic adaptation to pollution stress in *Daphnia* by comparing life history traits or analyzing effect concentration values of genotypes derived from habitats that differ in their exposure to pollutant load (e.g. Barata et al. 2002; Lopes et al. 2006; Coors et al. 2009; Agra et al. 2010). In the following section, an overview of the results of the research program on *Daphnia* is provided, integrating observations that have been presented elsewhere as individual case-studies (Coors and De Meester 2008, 2010; Coors et al. 2008, 2009; Jansen et al. 2010, unpublished observations).

## Carbaryl resistance in *D. magna* and its consequences

### Carbaryl selection experiment

To investigate to what extent natural populations can genetically adapt to the presence of a model pesticide, the *N*-methyl carbamate insecticide carbaryl was used in an experimental evolution approach. Previous experiments revealed that natural populations of *D. magna* inhabiting farmland ponds and other shallow ponds and lakes in Belgium differ in their sensitivity to carbaryl in relation to land-use intensity, suggestive of local genetic adaptation (Coors et al. 2009). We performed a selection experiment (Fig. 1), in which standardized samples of genotypes derived from the 10 natural populations studied by Coors et al. (2009) were exposed to standardized pulses of carbaryl (carbaryl treatment) or not (control) (Coors et al. unpublished). In brief, in the containers with carbaryl selection, *Daphnia* were exposed to three pulses of carbaryl (32 μg/l) that were given every 14 days. The containers were stocked with juveniles from clonal lineages obtained by hatching dormant egg banks of the 10 pond populations. Replicated outdoor containers were inoculated using a split-brood design for up to 125 clones per population. So each container received a standardized set of randomly selected clonal lineages (each represented by one individual per container) representing the genetic diversity of each of the natural populations. 26 days after the last carbaryl pulse, *D. magna* females were isolated from the containers and individually cultured as clonal lineages in the laboratory for several generations prior to the start of any further experiment, to obviate interference from maternal effects.

For each of the 10 natural populations, our approach yielded three selection groups: the start populations that were directly hatched from the natural dormant egg bank and kept as laboratory cultures (start-control group), populations derived from the containers that were not exposed to carbaryl (container-control group), and populations derived from the containers that were exposed to carbaryl (carbaryl-selected group) (Fig. 1). Both the start-control group and container-control group did not undergo carbaryl selection, yet the latter populations may have undergone selection imposed by the container environment. In subsequent life-table experiments, we worked with a subset of those groups to study changes in trait values in an effort to both study micro-evolutionary changes in the response to carbaryl (evolution of resistance) as well as costs of adaptation in carbaryl-free environments and in the presence of additional stressors.

In two life-table experiments, clones from the different selection groups were compared in the presence/absence of different stressors. In a first experiment, called the two-factor life-table experiment (Fig. 1), life history traits were analysed in the presence/absence of carbaryl and parasite spores. The second experiment, called the three-factor life-table experiment (Fig. 1), was performed with the same stressors and with fish kairomones. Information of both life-table experiments was used to evaluate microevolution of carbaryl resistance and evolutionary costs of this resistance.

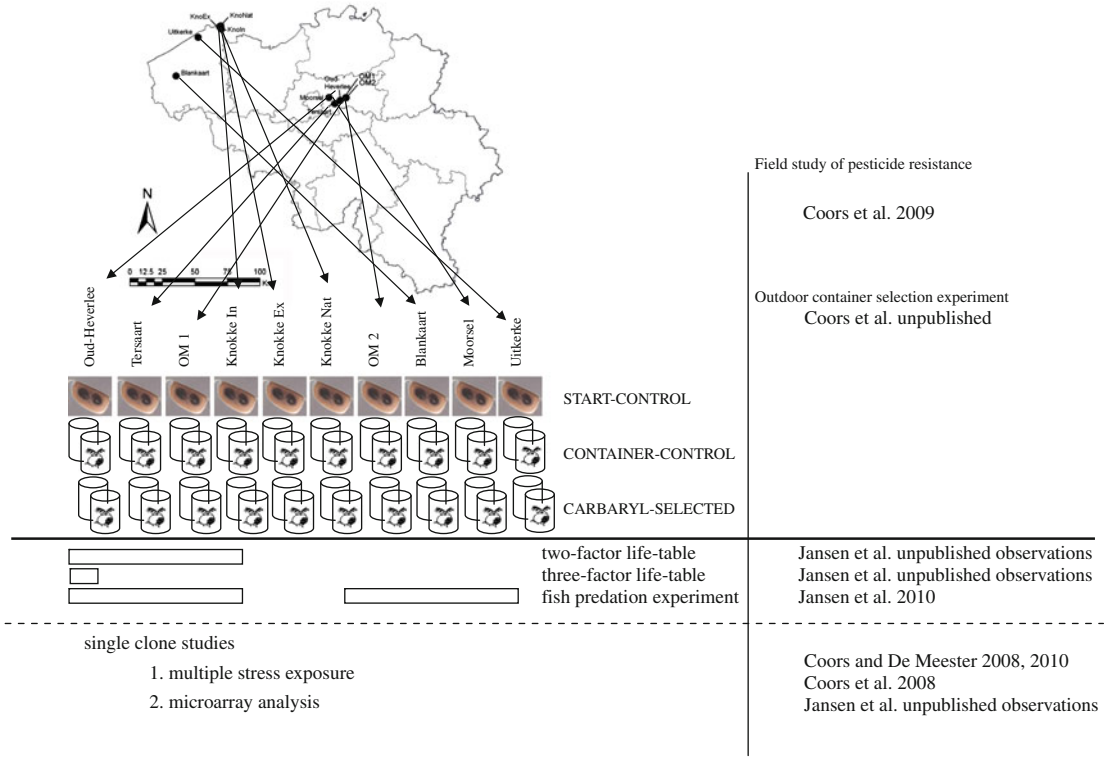


**Fig. 1** Overview of the research program integrating research on the evolution of pesticide resistance, its evolutionary costs and interactions with biotic stressors in a model system involving the water flea *Daphnia magna* and the pesticide carbaryl. Central is the carbaryl selection experiment with clones from 10 natural populations and the fish predation experiment and two life-table experiments with exposure to carbaryl and natural stressors (parasites and predation risk). Indicated are three selection groups obtained after the selection experiment: start-control group (clones that did not experience container selection), container-control group and carbaryl-selected group. The subset of selection groups and natural populations used in the experiments are indicated

## Microevolution of carbaryl resistance

The aim of the two life-table experiments was to evaluate whether clones from the carbaryl-selected group had evolved resistance by comparing key life history traits with those of clones from the start-control or container-control groups in the absence and presence of carbaryl. Contrast analyses between isolates from the container-control and start-control group did not reveal differences in key life history traits between both groups (Jansen et al. unpublished observations). This indicates that selection imposed by the container environment could be neglected.

Clear evidence of evolved resistance was observed in both life-table experiments. In the two-factor life-table experiment, eight different clones from each of the selection groups (start-control, container-control and carbaryl-selected) derived from four natural populations (Tersaart, Oud-Heverlee, Knokke In and OM 1) were exposed to four treatments in the laboratory: carbaryl (8 µg/l), parasite spores of the endoparasite *Pasteuria ramosa* ($500 \times 10^2$ spores/ml), a combination of both, or no stressor. Animals that previously experienced carbaryl (carbaryl-selected group) in the selection experiment showed a lower mortality than those from the control group after 4 days of exposure to carbaryl in the laboratory (Jansen et al. unpublished observations; Fig. 2a; carbaryl exposure × carbaryl selection: $F_{2,200} = 4.00$, $p < 0.05$). In the three-factor life-table experiment, four clones from the Oud-Heverlee start-control and carbaryl-selected groups were exposed to eight treatments in the laboratory: carbaryl (8 µg/l), parasite spores ($375 \times 10^2$ spores/ml), fish kairomones (three fishes *Leuciscus idus*/100 l), a control and all their combinations. Adaptive evolution of carbaryl resistance was seen for individual reproductive performance (quantified as the intrinsic rate of natural increase but ignoring mortality, as in Van Doorslaer et al. 2009) in the absence of fish kairomones (Jansen et al. unpublished observations; Fig. 2b; carbaryl selection × fish exposure × carbaryl exposure: $F_{1,182} = 5.90$; $p < 0.05$). While individual reproductive performance was reduced by carbaryl exposure in the start-control group, it was not affected in the carbaryl-selected group. Noteworthy, evolved resistance could only be detected in the absence of fish kairomones. In the presence of fish kairomones, carbaryl exposure did not affect performance in any selection group (Fig. 2b).

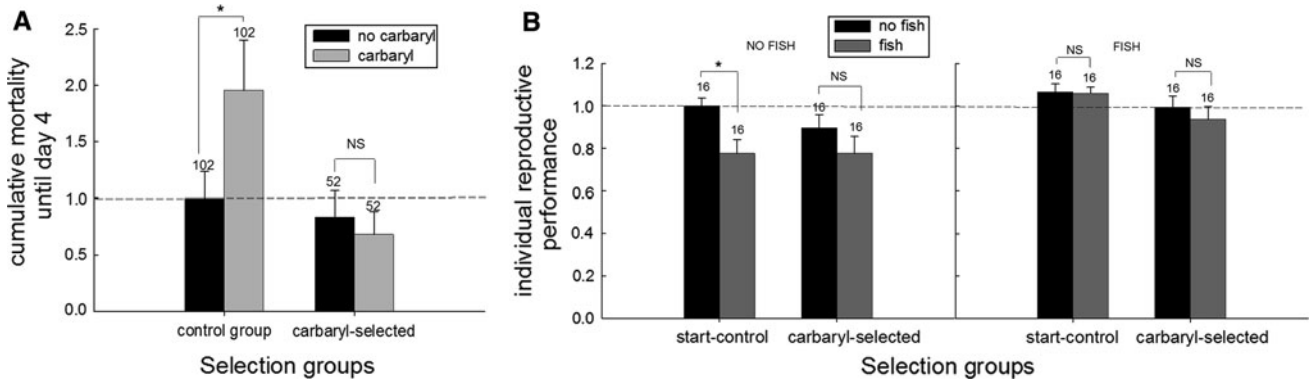


**Fig. 2** Microevolution of carbaryl resistance: an overview of the evidence **a** In the two-factor life-table experiment, cumulative mortality across the first 4 days of exposure to carbaryl was lower in the carbaryl-selected than in the control group (start-control and container-control together) (Jansen et al. unpublished observations). **b** In the three-factor life-table experiment, individual reproductive performance was not reduced upon exposure to carbaryl in the carbaryl-selected group in the absence of fish kairomones, while in the start-control group it was reduced compared to the control treatment without exposure to carbaryl. In the presence of fish kairomones, carbaryl exposure did not affect performance in any selection group. Means are given relative to the control treatment which is set to one. Significant differences are indicated by an *asterisk*

Both experiments showed that carbaryl resistance could evolve after exposure of *D. magna* populations to carbaryl. Carbaryl-selected clones from the selection experiment were more resistant to a new carbaryl pulse than clones never exposed to carbaryl before.

## Evolutionary costs of resistance

To examine costs of resistance, we used the data of the same two life-table experiments outlined above. In the two-factor life-table experiment, a cost of carbaryl selection was detected: clones of the carbaryl-selected group had an overall higher mortality compared to clones from the start-control populations (carbaryl selection: $F_{1,182} = 9.04$, $p < 0.05$). This was the case both in the presence and absence of pesticide exposure (carbaryl selection × carbaryl exposure: $F_{1,182} = 0.17$, $p > 0.05$), indicating that in this set of clones selection by carbaryl did result in an overall increased susceptibility to stress.

In the presence of parasites, we detected several costs of evolution. More specifically, we observed an increased susceptibility to parasites in both life-table experiments. In the two-factor life-table experiment, the individual reproductive performance of carbaryl-selected clones was more affected by parasite challenge than that of control clones (Fig. 3a; carbaryl selection × parasite exposure: $F_{1,199} = 4.22$, $p < 0.01$). The carbaryl-selected clones also tended to show a faster sterilization rate in the presence of parasite spores compared to control clones (Jansen et al. unpublished observations; Fig. 3b; carbaryl selection: $F_{2,67} = 2.44$, $p \sim 0.05$). This cost of carbaryl selection was also revealed in the three-factor life-table experiment in the presence of parasite exposure. Here, clones from the carbaryl-selected group suffered more from parasite infections compared to control clones. The difference between infection rates with immature and mature spores in both groups indicated that carbaryl-selected clones were less able to reduce the development rate of parasite spores (Jansen et al. unpublished observations; Fig. 3c, d). Infection intensities (spore load/individual) with mature spores were also highest in carbaryl-selected clones (Fig. 3e; carbaryl selection: $F_{1,59} = 6.96$, $p < 0.05$). Note that this type of costs constitutes "hidden" costs, as they would not become apparent when not tested in the presence of parasite spores. Both experiments thus showed that carbaryl-selected genotypes were more susceptible to parasite infections compared to non selected clones.

In an additional fish predation experiment, we tested for differences in consumption of carbaryl-selected and container-control clones by sticklebacks. For this, a total of 75 individuals were placed together in 30 l water and one stickleback was allowed to consume *Daphnia* (Jansen et al. 2010). Carbaryl-selected and container-control groups were tested separately and no cost of resistance in terms of increased vulnerability to visual fish predators (Jansen et al. 2010) was observed. Clones from the container-control and carbaryl-selected groups did not differ in their ability to avoid predation by stickleback (Jansen et al. 2010; carbaryl selection: $F_{1,31} = 1.69$; $p > 0.05$). Further, clones from the start-control and carbaryl-selected group did not differ in their life history response to fish kairomones in the three-factor life-table experiment (Jansen et al. unpublished observations; none of the effects involving fish were significant). In conclusion, while carbaryl-selected clones were more vulnerable to parasite infections, they did not carry a cost in terms of fish predation. This observation indicates a context-dependent cost of adaptation.

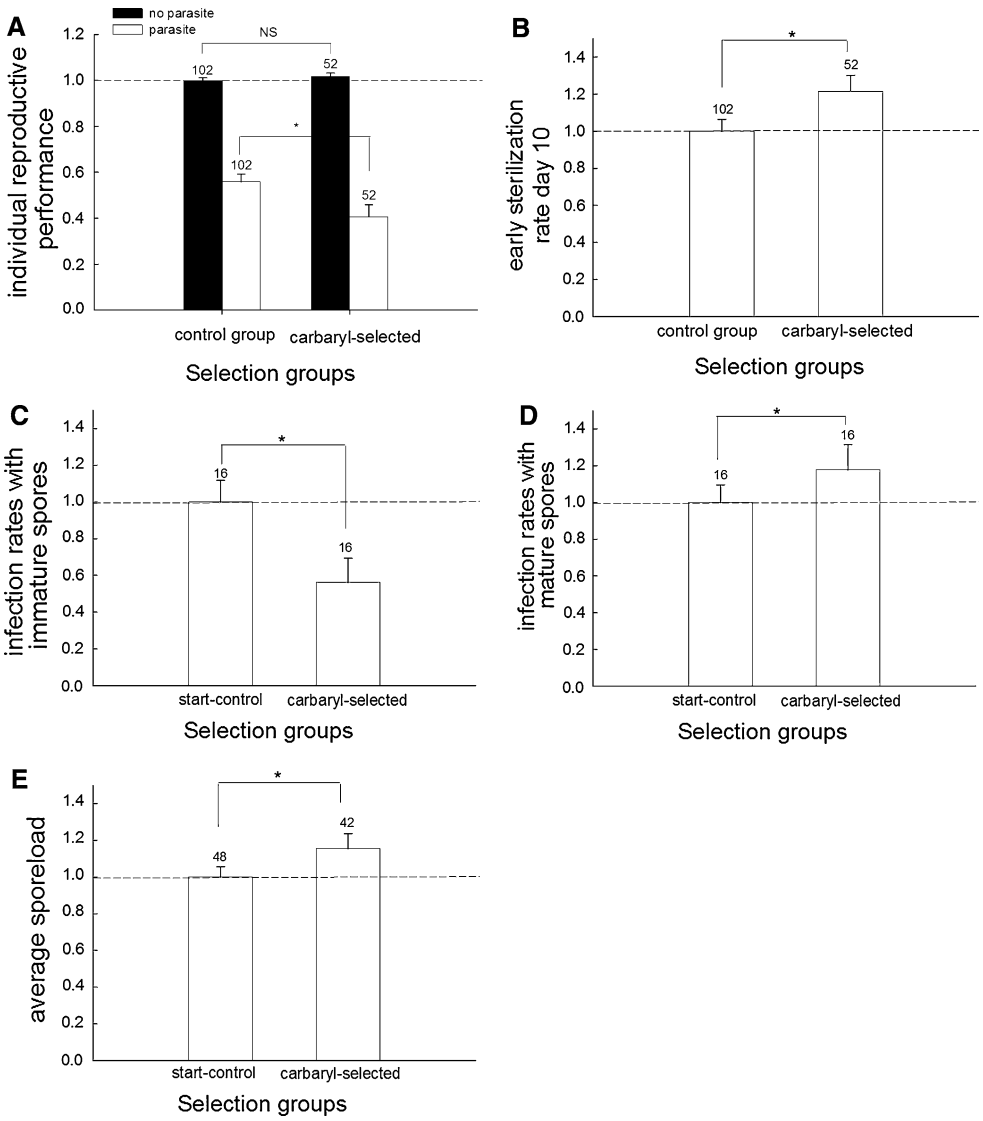


**Fig. 3** Costs of carbaryl selection after exposure to parasite spores as documented in the two- and three-factor life-table experiments (mean + 1 SE). When exposed to parasite spores, the carbaryl-selected group in the two-factor life-table experiment has **a** a lower individual reproductive performance and **b** an accelerated sterilization rate compared to the control groups (average start-control and container-control) (Jansen et al. unpublished observations). When exposed to parasite spores, the carbaryl-selected group in the three-factor life-table experiment has **c** a lower infection rate with immature spores but **d** a higher infection rate with mature spores and **e** higher loads of mature spores, indicating a lowered capacity to suppress spore development, than the control groups (Jansen et al. unpublished observations). Averages are given relative to the control treatment which is set to one

## Interactions between stressors

In both the two- and three-factor life-table experiments following the selection experiment, the presence of interactions between stressors was evaluated. Importantly, this approach not only allowed studying interactions between current stressors but also between current stress and past stress associated with carbaryl exposure in the carbaryl selection experiment. In the literature, there is a lack of information on interactions between past and current stressor combinations.

Overall, relatively few interactions between pesticide stress when applied together with other stressors were observed. More specifically, only strong interaction effects in the case of co-exposure to parasites could be determined. An interaction between exposure to carbaryl and to parasite spores for early sterilization (carbaryl exposure: $F_{1,67} = 10.80$, $p < 0.01$) was present. The percentage sterilized individuals on day 10 (early sterilization) when exposed to parasite spores is higher in the presence of the stressor carbaryl (two-factor life-table experiment; Jansen et al. unpublished observations). These synergistic effects between carbaryl and parasite exposure, in earlier studies documented for a single *Daphnia magna* clone (Coors and De Meester 2008, 2010; Coors et al. 2008), were thus shown to be quite generally present. Yet, the response is not general to the extent that it occurs in all clones.

Overall, interaction effects with fish predation risk tended to be absent except for an antagonistic interaction between carbaryl and fish shaping intrinsic population growth rate (carbaryl exposure × fish exposure: $F_{1,182} = 4.40$, $p < 0.05$). In the absence of fish kairomones, the intrinsic population growth rate was reduced under carbaryl exposure. This reduction was, however, cancelled out if animals were exposed to fish kairomones and carbaryl stress at the same moment. The antagonistic interaction

between fish exposure and carbaryl was also present for individual reproductive performance. Yet here the interaction was more specific, as it only occurred in animals not exposed to carbaryl stress in the past (Jansen et al. unpublished observations).

The results of all here discussed experiments involving different stressors (Coors et al. 2008; Coors and De Meester 2008, 2010; Jansen et al. unpublished observations) underscore that prudence is called for when extrapolating laboratory results to real field situations. First, generalization of observed effects of single stressors sometimes may lead to under- or overestimation of risks under field situations (Relyea 2006). Secondly, similar to the variation in interaction effects reported in the work of Relyea (Relyea and Mills 2001 versus Relyea 2003), the combined experiments on *Daphnia* as reported on here also show some variation in interaction effects among studies, stressors and *Daphnia* genotypes.

Several interactions were detected between past exposure to carbaryl (selection experiment) and the response of *Daphnia* to single stressors and combinations of stressors. As mentioned under the section on costs of selection, past exposure to carbaryl affected several infection-related variables, including an increased susceptibility to parasites. Furthermore, past exposure to carbaryl also affected the interaction between current exposures to stressors. Current exposure to carbaryl interacted with fish kairomones in the control group but not in the group of animals that were exposed to carbaryl in the selection experiment (Fig. 2b). These findings indicate that ignoring past exposure associated with evolved resistance may result in unexplained variation among clones in their response to single and combined stressors.

## Mechanistic base of resistance, costs and interactions: first insights and perspectives

The patterns of carbaryl resistance documented in the here described research, its evolutionary costs and interactions between biotic stressors and both current and past exposure to carbaryl still need to be fully unraveled at the underlying chemical/physiological level. So far, we only started generating some possible mechanistic scenarios, which are partly supported by a cDNA *D. magna* microarray analysis of animals exposed to single stressors (Jansen et al. unpublished observations). Earlier studies showed the usefulness of microarray chip technology in revealing the genetic basis of DDT resistance in *D. melanogaster* (Denholm et al. 2002). In the analyses, differently expressed transcripts under direct exposure to carbaryl, parasite spores and fish kairomones were observed and quantified comparing exposed and non-exposed individuals. One of the most important results from the life-table experiments is the increased susceptibility to the parasite *P. ramosa* in the presence of current carbaryl stress (synergistic interaction) and after carbaryl selection (cost of evolution). A first potential mechanism for these observations is that both current carbaryl exposure and carbaryl selection resulted in a weakened host immune system. Immunosuppression under direct exposure to pollutants is a well-known phenomenon (e.g. Albert et al. 2007; Aggarwal et al. 2008). A possible pathway would be through reduced activity of phenoloxidase, a key immune component in *Daphnia* (Pauwels et al. 2010) shown to be less present under pesticide exposure (Campero et al. 2008). Further, carbaryl may weaken the carapax, the physical barrier of the host, and a major defence against parasite spores (Auld et al. 2010). The idea of a decrease in carapax strength is supported by our microarray analysis, revealing a downregulation of gene fragments related to cuticle proteins under direct carbaryl exposure (Jansen et al. unpublished observations). Finally, carbaryl selection may have resulted in clones with reduced immunocompetence. For instance, as direct exposure of carbaryl decreases the transcription of gene fragments related to cuticle, carbaryl-selected genotypes could be those increasing the transcription of these genes at the expense of immune defence.

There are two complementary approaches to gain further understanding of the mechanistic base of the responses detailed in this review. On the one hand, the microarray analyses should be further strengthened by input of new information and should be extended from single stressor to multiple stressor microarrays. The observed transcription patterns under single stressor exposure were non-conclusive with respect to the mechanistic base of the synergistic effects among stressors. The already selected fragments, which are either up- or downregulated by microarray analysis under single stressor exposure, should be studied in more detail by real-time quantitative polymerase chain reaction (qRT-PCR). Poynton et al. (2007) observed a downregulation of immune related fragments by coper exposure. This downregulation was observed after microarray analysis and confirmed by qRT-PCR, showing the potential of both techniques. The release of the full *D. magna* genome (see Colbourne et al. 2011 for the *D. pulex* genome) and the annotation of it, hopefully in the near future, will be of crucial importance for a more complete understanding of the mechanistic base of responses to single and combined stressors and their variation among populations of *D. magna*. A full coverage of the genome and a full transcriptome array will be needed to obtain more insight into the different underlying mechanisms. At the same time, more studies of phenotypic responses are also needed. These should involve direct quantification of key components of the immune defence system, like the activity of phenoloxidase and the strength/thickness of the

physical barrier (carapax) under direct carbaryl exposure and after carbaryl selection.

## Conclusions

The basic findings of the here proposed integrated research on evolutionary ecotoxicology using the water flea *Daphnia* as a model system were that (1) carbaryl selection in genetically standardized *Daphnia* populations can result in rapid evolution of carbaryl resistance, which is associated with evolutionary costs, and that (2) synergistic interactions occurred between carbaryl and parasite exposure but not between carbaryl and fish kairomones.

Our observations of a context-dependent evolved resistance to carbaryl and a strong variability in life history traits after single and combined exposure in combination with a past stressor lead to the conclusion that past exposure to pesticides is important and cannot be neglected when evaluating responses to current stressors. So far, the relationships between evolved resistance, its evolutionary costs and exposure to current stressors remain largely unpredictable. The most promising way to advance both key research lines in ecotoxicology and to ultimately arrive at a predictive framework for these interactions is to further unravel their mechanistic basis. The performed microarray analyses on single stressor-exposed animals provided a first step to achieve this, but should be complemented by multiple exposure analyses and measurements of key components of the immune system.

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