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INTERACTION BETWEEN STRESS INDUCED BY COMPETITION, PREDATION, AND AN INSECTICIDE ON THE RESPONSE OF AQUATIC INVERTEBRATES

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Abstract: The present study investigated the effects of species interactions like competition and (intraguild) predation on the sensitivity of aquatic organisms to the insecticide chlorpyrifos. In the first experiment, combined effects of chlorpyrifos and different levels of intraspecific and interspecific interaction were assessed on *Gammarus pulex* survival using *Asellus aquaticus* as an interacting species. Intraspecific and interspecific interactions increased the time to extinction of *G. pulex* up to a factor of 2, most likely because of the cannibalistic nature of *G. pulex* and its intraguild predation on *A. aquaticus* under stress conditions. In the second experiment, combined effects of chlorpyrifos and intraspecific and interspecific interaction were assessed on *Daphnia pulex* abundance using *Brachionus calyciflorus* as a competing species and *Chaoborus* sp. larvae as a predator. Intraspecific and interspecific interactions significantly affected the *D. pulex* population structure, but they did not influence the total population size. Predation decimated *D. pulex* abundance; however, interacting effects of predation and chlorpyrifos exposure were less noticeable at high exposure concentrations because of the reduced predatory efficiency of *Chaoborus* sp. larvae. The present study shows that species interactions do not always increase the vulnerability of aquatic populations to chemical stress and that some interactions (e.g., cannibalism and intraguild predation) or reduced predator grazing pressure can alleviate competition and predation stress on population-level insecticide effects under food-limiting conditions. *Environ Toxicol Chem* 2017;36:2485–2492. © 2017 SETAC

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INTRODUCTION

Existing ecological risk-assessment procedures for chemicals are, for the most part, based on the use of threshold concentrations derived with single-species toxicity data to protect ecosystems [1]. Such an approach often disregards side effects caused by chemical pollution as a result of ecological interactions. Species interactions such as competition or predation are susceptible to chemical stress (e.g., Foit et al. [2], Del Arco et al. [3,4]), possibly leading to population or community changes and impacts on several ecosystem functions and processes (e.g., nutrient cycling, food chain provision). The literature shows that intraspecific competition can increase the sensitivity of insect larvae to insecticides (e.g., Liess [5]), result in higher population survival rates as a result of increased availability of resources (e.g., Postma et al. [6]), or temporarily affect the structure of aquatic populations [4]. Next to sensitivity, intraspecific competition may also influence population fitness and the recovery potential of populations after a chemical stress event. For instance, Liess and Foit [7] showed that *Daphnia magna* exposed to the insecticide fenvalerate experienced delayed population recovery, whereby the level of intraspecific competition determined the length of time needed for the recovery of population structure. Laboratory experiments have shown that interspecific competition may lead to population decreases in less fitted species or in species with the highest sensitivity to the toxicant [2], whereas others have observed an adaptive behavior encompassing changes in feeding strategies to overcome food resource limitations [3]. As is often seen in microcosm and mesocosm experiments, interspecific competition generally leads to an increase of the least sensitive competing species because of increased availability of resources [8] and a slower recovery of the most sensitive one [2]. Such a situation may persist in time, consequently leading to significant effects on the structure of biological communities.

Predation is often considered to result in more predictable effects on aquatic populations exposed to toxicants than competition alone. Predators may affect prey populations in ways competition cannot: by consuming prey (density-mediated interactions) and by inducing costly defensive strategies (trait-mediated interactions). Although not studied directly with insecticides or other potentially toxic substances, trait-mediated indirect effects (the costs of antipredator defenses) can be equal to or stronger than density-mediated indirect effects (the direct and indirect interactions from predator consumption) [9]. Predators and insecticides are quite similar in the sense that both consumption and toxicity reduce population density, but they differ in adaptivity and selectivity. Some organisms may also feed on their competitors for a shared resource (e.g., food), which is called "intraguild predation."

The objective of the present study was to gain understanding on how the sensitivity of aquatic individuals and populations is influenced by ecological interactions such as intraspecific and interspecific competition and predation in the presence of a pesticide. The hypothesis is that any form of competition over food will enhance the sensitivity of species because of a reduced fitness, whereas predation may decrease or enhance the sensitivity depending on whether the focal species is the

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predator or the prey, respectively. This was done by selecting sets of species that often occur together and are seen either as each other's competitors over food or as having a strong predator–prey interaction. Two sets of species were selected. The first set was formed by the amphipod *Gammarus pulex*, which competed over food resources with the isopod *Asellus aquaticus*, also showing intraguild competition [3]. The second set of species was formed by the cladoceran *Daphnia pulex* (focal species), a rotifer that competed over the same food resource, and an external predator that predated on *D. pulex*. The experiments were performed with several levels of the organophosphate insecticide chlorpyrifos. Chlorpyrifos was chosen because detailed toxicity data were available for all individual species. Experiments were performed under various strengths of intraspecific and interspecific interactions and predation. Combined effects of species interactions and the insecticide were evaluated on the survival of *G. pulex* individuals and on the size and structure of the *D. pulex* population at various time points.

MATERIALS AND METHODS

Experiment with G. pulex

In the first experiment, the combined effects of chlorpyrifos and the stress caused by intraspecific and interspecific interaction were studied on the survival of *G. pulex* (Table 1). The experiment was designed using *A. aquaticus* (Isopoda) as competing species but also a species on which *G. pulex* exerts intraguild predation, and using poplar leaves as a food source. *Gammarus pulex* individuals were collected from a pond (Renkum, The Netherlands, 51°58′9.31″N, 5°48′9.88″E) located in the Duno estate, which is managed by the Gelders landscape and castles foundation and where no pesticides are used. Individuals were allowed to acclimatize to laboratory conditions for several days prior to the experiment. After acclimatization, *G. pulex* individuals were stocked in 72 glass jars (1.5 L) and placed in a water bath at 15 ± 1 °C. High-pressure metal halide lamps (Philips HPI-T, 400 W) were used to provide a daily photoperiod of 12 h, with a light intensity of approximately 500 μE/m^{2}s at the jar's water surface. Each jar contained 3.5 ± 1.1 mg (mean ± standard deviation [SD], $n = 360$) of preweighed autumn-shed poplar (*Populus nigra* L.) leaf disks. The food source and amount of food provided were determined based on results of the pretest described in the Supplemental Data. Poplar leaf disks were prepared by carefully avoiding the midrib of the leaf and conditioned for 10 d by soaking in filtered pond water to accumulate microbiological food substrate. Each jar contained a stainless steel mesh rectangular structure and was filled with 1 L of water collected from a pond located at Wageningen University campus, where no pesticides are used (Wageningen, The Netherlands, 51°59′16″N, 5°40′5″E), filtered through a 20-μm phytoplankton net. Water and leaf disks were refreshed every 7 d. The experiment with *G. pulex* was performed in triplicate ($n = 3$) with 3 levels of intraspecific interaction (IA-control, IA-low, and IA-medium), 4 levels of interspecific interaction (IR-control, IR-low, IR-medium, and IR-high), and 4 different chlorpyrifos treatments (CPF-control, CPF-low, CPF-medium, and CPF-high) and had a duration of 16 d. Controls were shared between the intraspecific and interspecific treatments and stocked with 5 *G. pulex* individuals. Low and medium intraspecific treatments consisted of 10 and 15 *G. pulex*, respectively (Table 1). Low, medium, and high interspecific treatments consisted of 5 *G. pulex* and 5, 10, and 15 *A. aquaticus*, respectively (Table 1), sampled from the same pond from which the *G. pulex* individuals were collected. Chlorpyrifos (O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, 99% purity, CAS no. 2921-88-20) purchased from Dr. Ehrenstorfer was applied once a week at concentrations of 0 (controls), 0.15, 0.20, and 0.25 μg/L for 2 wk. Insecticide concentrations were chosen according to the *G. pulex* 96-h 0, 10, 30, and 50% effective concentration (EC0, EC10, EC30, and EC50, respectively) values obtained from the dose–response curve provided by Rubach et al. [10], with the intent of imposing chemical stress leading to a partial mortality or immobility response. The insecticide was applied by spiking aliquots of a chlorpyrifos stock solution of 480 mg/L on the water surface of the test unit and then stirring for 5 s.

Competition for the food resource began 2 d prior to the first insecticide application. *Gammarus pulex* and *A. aquaticus* survival was monitored prior (day –2) to and 2, 4, 7, 11, and 14 d after the first chlorpyrifos application. A 10-mL aliquot of the exposure medium was sampled from all replicates of each chlorpyrifos concentration 1 h after insecticide application and prior to the exposure media exchange to verify chlorpyrifos exposure concentrations and to assess the degradation of chlorpyrifos during the experimental period. Additional environmental parameters (temperature, pH, dissolved oxygen concentration, and conductivity) were measured weekly at the beginning of the photoperiod. Temperature, pH, and dissolved oxygen concentration were measured with a WTW 340i multimeter, and conductivity was measured with a WTW 315i meter (Supplemental Data, Table S1).

Table 1. Experimental setup summary showing the tested species' interactions at various interaction levels in the *Gammarus pulex* and *Daphnia pulex* experiments[a]

	G. pulex		*D. pulex*		
Species interaction	Intraspecific interaction	Interspecific interaction	Intraspecific interaction	Interspecific interaction	Predation
Control	5 *G. pulex*	5 *G. pulex*	10 *D. pulex*	10 *D. pulex*	40 *D. pulex*
Low	10 *G. pulex*	5 *G. pulex* + 5 *A. aquaticus*	20 *D. pulex*	10 *D. pulex* + 333 *B. calyciflorus*	40 *D. pulex* + 1 *Chaoborus* larvae
Medium	15 *G. pulex*	5 *G. pulex* + 10 *A. aquaticus*	40 *D. pulex*	10 *D. pulex* + 666 *B. calyciflorus*	—
High	—	5 *G. pulex* + 15 *A. aquaticus*	—	10 *D. pulex* + 999 *B. calyciflorus*	—

[a]Each interaction/predation treatment was tested against 4 chlorpyrifos concentrations. A dash (—) indicates that the treatment was not evaluated.
A. aquaticus = *Asellus aquaticus*; *B. calyciflorus* = *Brachionus calyciflorus*.

Experiment with D. pulex

In the second experiment, the combined effects of chlorpyrifos and the stress caused by intraspecific and interspecific interaction were studied on the population structure of *D. pulex* (Table 1). The experiment was designed using *Brachionus calyciflorus* (Rotifera) as a competing species, *Chaoborus* sp. (larval phantom midges) as a predator, and *Scenedesmus obliquus* (green algae) as food source. Both *D. pulex* and *S. obliquus* were obtained from laboratory cultures at Wageningen University and grown in modified WC medium [11] at room temperature. *Brachionus calyciflorus* cysts were obtained from MicroBioTests and grown in WC medium at 20 ± 1 °C in flasks fed with ample concentrations of *S. obliquus* (approximately 1 million cells/mL daily). *Chaoborus* sp. were collected in February 2014 from previously noncontaminated ditches at the Sinderhoeve Experimental Station (Renkum, The Netherlands, 51°59′54.77″N, 5°45′8.33″E) and allowed to acclimatize to experimental temperature and diet (WC medium at 20 °C) over 4 d. Such acclimatization was achieved by replacing one-fourth of the containment bucket's water every day with WC medium and by feeding *Chaoborus* sp. individuals with *D. pulex* ad libitum. During the experiment, if *Chaoborus* sp. individuals died or pupated, they were replaced within 24 h.

The experiment was conducted using 96 glass jars (1.5 L) filled with 1 L of WC medium and placed in a water bath at 20 ± 1 °C with a 1-h photoperiod under low artificial fluorescent lighting (15–25 μE/m^{2}s). The experiment lasted for 24 d and was performed in triplicate ($n = 3$) with 3 levels of intraspecific interaction (IA-control, IA-low, and IA-medium), 4 levels of interspecific interaction (IR-control, IR-low, IR-medium, and IR-high), and 2 predation treatments (P-control and P-low), all under 4 different chlorpyrifos treatments (CPF-control, CPF-low, CPF-medium, and CPF-high; Table 1). Species interaction controls for the intraspecies and interspecies interaction tests were set up with 10 *D. pulex* individuals, whereas controls for the predation test were set up with 40 *D. pulex* individuals. Low and medium interaction treatments of the intraspecific test were established by stocking 20 and 40 *D. pulex* individuals, respectively. Low, medium, and high interspecific interaction treatments were set up by adding approximately 333, 666, and 999 rotifers, respectively, to test units containing 10 *D. pulex* (Table 1). Low predation treatment was set up by adding one *Chaoborus* sp. larvae to the test units. In all test units, the initial *D. pulex* population consisted of 20% adults (>0.8 mm) and 80% subadults (0.6-0.8 mm). Each jar received a daily dose of concentrated algae (*S. obliquus*) consisting of approximately 8.5 and 34 million cells/mL (equivalent to 1 g/C in 20 *D. pulex*) for the interaction and the predation test units, respectively. A side test showed that interspecific interaction treatments received approximately 1.4 million *S. obliquus* cells/mL extra during *B. calyciflorus* inoculation as a result of uneaten food from *B. calyciflorus* stock cultures (data not shown).

Test organisms underwent 3 d of interaction prior to chlorpyrifos exposure. Chlorpyrifos was applied once a week for a period of 3 wk at the following nominal concentrations: 0 (control), 0.10, 0.40, and 0.75 μg/L. Exposure concentrations represented the EC0, EC10, EC30, and EC50 for *D. pulex*, calculated from a dose–response curve obtained from Van der Hoeven and Gerritsen [12]. Chlorpyrifos was applied by spiking aliquots of a stock solution of 480 mg/L on the water surface of the test unit and stirring for 5 s. Survival of *D. pulex* was monitored prior to the first chlorpyrifos application (day –3) and 2, 4, 7, 14, and 21 d after the first insecticide application. While monitoring survival, *D. pulex* individuals were separated into age classes using sieves with different mesh size (>800 μm for adults, 600 to 800 μm for subadults, and 200 to 600 μm for juveniles), counted alive, and returned to their glass jars. To minimize organism disturbance during sampling sessions and reduction of the experimental media volume, 12 additional jars were set up to monitor chlorpyrifos exposure concentrations and degradation during the experimental period as well as water quality parameters. In these test units, water samples were taken to monitor the chlorpyrifos concentration 1 h and 7 d after insecticide application (just before the next application). Temperature, pH, dissolved oxygen concentration, and conductivity were measured weekly at the beginning of the photoperiod using the same devices as described in the *G. pulex* experiment (Supplemental Data, Table S1).

Chlorpyrifos analysis

Exposure media samples collected for chlorpyrifos analysis were added to glass test tubes containing 1 mL of *n*-hexane and gently shaken by hand for 1 min. Afterward they were shaken at 175 rpm for 15 min with a shaker table. The supernatant was pipetted into airtight glass injection vials and stored in the dark at 4 °C until further analysis. Chlorpyrifos was measured by splitless injection of 3 μL of the organic solvent solution in an Agilent 6890™ gas chromatograph equipped with an electron capture detector, according to the method described by Rubach et al. [10]. External standards of 0.5 to 10 μg/L chlorpyrifos were used to correct and control for extraction losses.

Data analysis

The effects of the insecticide exposure, the interaction/predation treatment, and their interaction on evaluated endpoints were analyzed using generalized linear models (GLMs) with GenStat, 17th edition (VSN International). Generalized linear models for the *G. pulex* experiment were constructed for each sampling day using abundance as the response variable and levels of chlorpyrifos and intraspecific and interspecific interaction as categorical predictor variables, as well as a binomial distribution with a logit link function. Furthermore, the 50% lethal concentration (LC50) for *G. pulex* was calculated for each interaction treatment and for each sampling day using a log-logistic regression model according to methods described by Rubach et al. [10] and measured chlorpyrifos concentrations after spiking.

Generalized linear models for the *D. pulex* experiment were constructed using total, adult, subadult, and juvenile abundance as the response variable and levels of chlorpyrifos, intraspecific and interspecific interaction, and predation as categorical predictor variables for each sampling day, using a Poisson distribution with logarithm as link function. The intercept of the calculated GLMs for *D. pulex* were used as a proxy to observe dynamics of the population size over time and to identify the carrying capacity of experimental units.

For both experiments, the effect of insecticide concentration, the interaction or predation treatment, or their interaction on the evaluated end point was considered to be significant when the calculated p values were <0.05 and moderately significant when they were between 0.05 and 0.10.

RESULTS AND DISCUSSION

Chlorpyrifos exposure

Measured chlorpyrifos concentrations in the *G. pulex* experiments were approximately 62% of the nominal

Table 2. Results of the chlorpyrifos analysis and calculated dissipation rate of chlorpyrifos in the *Gammarus pulex* and *Daphnia pulex* experiments (mean ± standard deviation, $n = 9$)[a]

Species	Interaction	Treatment	Nominal conc. (μg/L)	Measured conc. after application (μg/L)	7-d average conc. (μg/L)	7-d % dissipation (%)
G. pulex	Intraspecific/ interspecific	Low (EC10)	0.15	0.09 ± 0.01	0.07 ± 0.03	51 ± 12
		Medium (EC30)	0.20	0.11 ± 0.01	0.09 ± 0.03	52 ± 0.4
		High (EC50)	0.25	0.18 ± 0.03	0.14 ± 0.06	48 ± 5.8
D. pulex	Intraspecific/ interspecific	Low (EC10)	0.10	0.09 ± 0.02	0.07 ± 0.03	51 ± 6.9
		Medium (EC30)	0.40	0.37 ± 0.08	0.28 ± 0.11	48 ± 5.5
		High (EC50)	0.75	0.75 ± 0.16	0.55 ± 0.24	46 ± 6.4
D. pulex	Predation	Low (EC10)	0.10	0.11 ± 0.03	0.09 ± 0.03	65 ± 5.1
		Medium (EC30)	0.40	0.46 ± 0.11	0.38 ± 0.12	63 ± 6.9
		High (EC50)	0.75	0.85 ± 0.18	0.71 ± 0.20	68 ± 7.2

[a]On average, 56% of the applied dose of chlorpyrifos dissipated over 7 d.
ECx = x% effective concentration.

concentration, whereas measured concentrations in the *D. pulex* experiment were, on average, 103% of the nominal concentration. Nearly half of the applied chlorpyrifos dose dissipated from the experimental media 7 d after application (Table 2; Supplemental Data, Figure S1). Dissipation of chlorpyrifos from the test units was found to be within the ranges reported by Rubach et al. [10] for 4-d single-species toxicity tests performed with *G. pulex* (42–78%), *A. aquaticus* (60–61%), and *D. magna* (36–38%).

Effects of chlorpyrifos

Mortality in the chlorpyrifos and species interaction controls of the *G. pulex* experiment was only observed after day 11 and reached 13 ± 12% (average ± SD.) at the end of the experimental period (day 14). Chlorpyrifos exposure significantly reduced the survival of *G. pulex* 4 d after the first insecticide application (Table 3) and resulted in a total extinction of individuals in the interaction controls with high chlorpyrifos concentrations on day 7 (IA-control and IR-control in Figure 1). The calculated LC50 4-d values (0.11–0.14 μg/L; Table 4) in the interaction controls were found to be between values reported in other studies (i.e., 0.07 μg/L by Van Wijngaarden et al. [13] and 0.23 μg/L by Rubach et al. [10]).

Overall, the *D. pulex* population size increased during the experimental period until day 7, reaching a maximum density of 150 to 530 individuals/L in the interaction and predation controls (P-control in Figure 2). This was also shown by the increase of the GLM intercept up until day 14 (data not shown), suggesting that on this day the population reached the system's carrying capacity. Chlorpyrifos effects in the total population size of the interaction controls were only noticeable visually in the 0.40 and 0.75 μg/L treatments after the second application (Figure 2). Subadult and juvenile abundances showed a significant treatment-related decrease, whereas effects on adult abundance were less noticeable (Supplemental Data, Table S2 and Figures S2–S4). These results are in line with those shown by Van der Hoeven and Gerritsen [12], who reported early life stages of daphnids being more sensitive to chlorpyrifos than adult stages.

Effects of intraspecific interaction

Mortalities of *G. pulex* in intraspecific treatments without pesticide exposure were similar to those observed in controls at the end of the experiment, indicating that the levels of intraspecific interaction used in the present study did not result in dramatic survival effects (Figure 1a and Table 3).

The *D. pulex* population size in different intraspecific treatments tended to be similar toward the end of the experimental period, approaching a similar carrying capacity on day 7. However, a significantly higher total abundance was

Table 3. Significance of the predictor variables (chlorpyrifos, intraspecific and interspecific competition, predation, and interactions) and the interactions between chlorpyrifos and competition/predation in the *Gammarus pulex* and *Daphnia pulex* experiments at each time point as estimated by generalized linear models[a]

		Mortality of *G. pulex*						Total *D. pulex* abundance					
Treatment	Variable (stressor)	0 d	2 d	4 d	7 d	11 d	14 d	0 d	2 d	4 d	7 d	14 d	21 d
Intraspecific (IA)	Chlorpyrifos	NS	NS	<0.001	<0.001	<0.001	<0.001	NS	NS	NS	NS	<0.001	<0.001
	IA competition	NS	NS	0.02	0.01	NS	NS	<0.001	<0.001	0.001	0.06	0.07	<0.001
	Interactions	NS	NS	0.03	0.008	0.005	NS	NS	0.03	NS	NS	NS	NS
Interspecific (IR)	Chlorpyrifos	NS	NS	<0.001	<0.001	<0.001	<0.001	NS	NS	0.05	0.02	<0.001	<0.001
	IR competition	NS	0.04	<0.001	<0.001	<0.001	0.005	0.09	0.003	<0.001	<0.001	<0.001	<0.001
	Interaction	NS	0.008	0.01	0.004	0.001	0.004	0.09	NS	NS	NS	NS	NS
Predation	Chlorpyrifos	—	—	—	—	—	—	NS	NS	NS	NS	0.005	0.01
	Predation	—	—	—	—	—	—	NS	<0.001	<0.001	<0.001	<0.001	<0.001
	Interaction	—	—	—	—	—	—	NS	<0.001	0.002	0.002	NS	NS

[a]A dash (—) indicates that the treatment was not evaluated.
NS = not significant ($p \geq 0.1$).

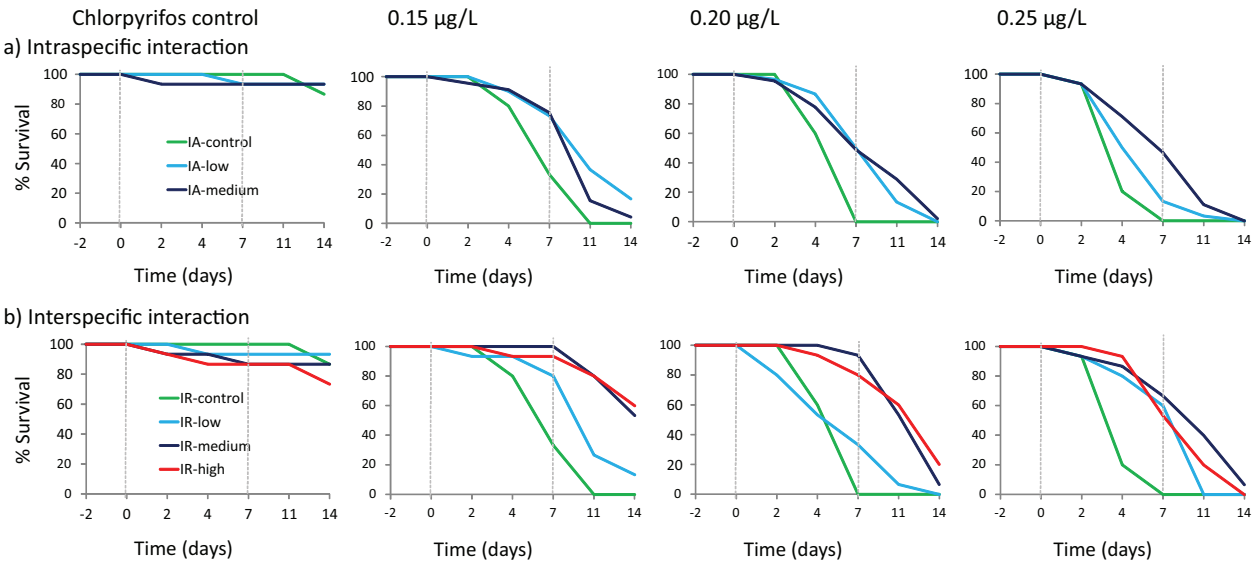

Figure 1. Results of the intraspecific interaction (**a**) and interspecific interaction (**b**) experiments performed with *Gammarus pulex* for the different chlorpyrifos treatments (control, 0.15, 0.20, and 0.25 µg/L). Dotted lines indicate chlorpyrifos applications. IA = intraspecific; IR = interspecific.

found in the intraspecific interaction controls at the end of the experiment (Figure 2a and Table 3). This is explained by the higher number of juveniles in interaction controls, indicating potentially higher population fitness and reproductive success in the systems with lower initial (sub-)adult densities (Supplemental Data, Figure S3).

Effects of interspecific interaction

Interspecific interaction treatments significantly decreased survival of *G. pulex* over time, with highest mortalities observed in the highest interaction treatment (Figure 1b and Table 3) at the end of the experimental period. The mortality increase caused by the highest interspecific interaction treatment was, although statistically significant, only 7% higher than in the interaction control.

On the contrary, the total population size of *D. pulex* was found to significantly increase with interspecific interaction (Figure 2a and Table 3). The increase was associated with the high abundance of adults and juveniles in test units with the highest *B. calyciflorus* densities (Supplemental Data, Figure S4). Similar findings were reported by Del Arco et al. [4], who attributed increasing population size of *D. magna* in the presence of *B. calyciflorus* to the consumption of this small rotifer, thus overcoming food limitation pressure. It is believed that a similar situation occurred in the present experiment.

Effects of predation on D. pulex

Effects of predation were much greater on *D. pulex* population size than intraspecific or interspecific interaction. Immediate reductions in abundance were observed after the addition of *Chaoborus* sp. larvae (after day 0; Figure 2) compared to the predation control. These abundance decreases continued for the remainder of the experiment in the total population, particularly in the adult and subadult age classes (Table 3; Supplemental Data, Table S2). Viaene et al. [14] found the largest effects of predation in juvenile and subadult *D. magna*, whereas in the present study juvenile *D. pulex* were the least affected and the only age class not to become extinct (Supplemental Data, Figure S5). This could be attributed to size differences between these 2 species, with adult individuals of *D. pulex* being smaller than those of *D. magna*. By the fourth day after the *Chaoborus* sp. larvae addition, adults and subadults had been completely consumed, and the average

Table 4. Calculated *Gammarus pulex* LC50 values (±95% confidence interval) based on time-allotted average exposure concentrations (µg/L) 4, 7, 11, and 14 d after chlorpyrifos exposure[a]

Treatment	Level	Day 4	Day 7	Day 11	Day 14
Intraspecific	Control	0.14 (0.12–0.18)	0.10 (0.10–0.10)	<<0.10[b]	<<0.10[b]
	Low	0.22 (0.17–0.29)	0.13 (0.11–0.16)	0.08 (0.07–0.11)	0.10 (NC)
	Medium	0.43[c] (0.15–1.3)	0.20 (0.13–0.32)	0.04 (0.02–0.06)	0.06 (0.02–0.25)
Interspecific	Control	0.11 (0.09–0.14)	0.07 (0.06–0.07)	<<0.07[b]	<<0.07[b]
	Low	0.23[c] (0.07–0.79)	[d]	0.05 (0.03–0.09)	<<0.07[b]
	Medium	[d]	[d]	0.14 (0.10–0.20)	0.07 (0.06–0.10)
	High		0.16 (0.13–0.20)	0.13 (0.11–0.15)	0.11 (NC)

[a]Values for interaction control and interspecific control differ because the average measured concentrations over all test vessels belonging to the same chlorpyrifos treatment were used.
[b]Not enough data points between the control and the lowest exposure concentration.
[c]Lethal concentration is greater than the highest tested concentration. LC50 was extrapolated.
[d]Not calculated, not enough mortality to fit a dose–response curve.
LC50 = 50% lethal concentration; NC = no confidence intervals could be calculated.

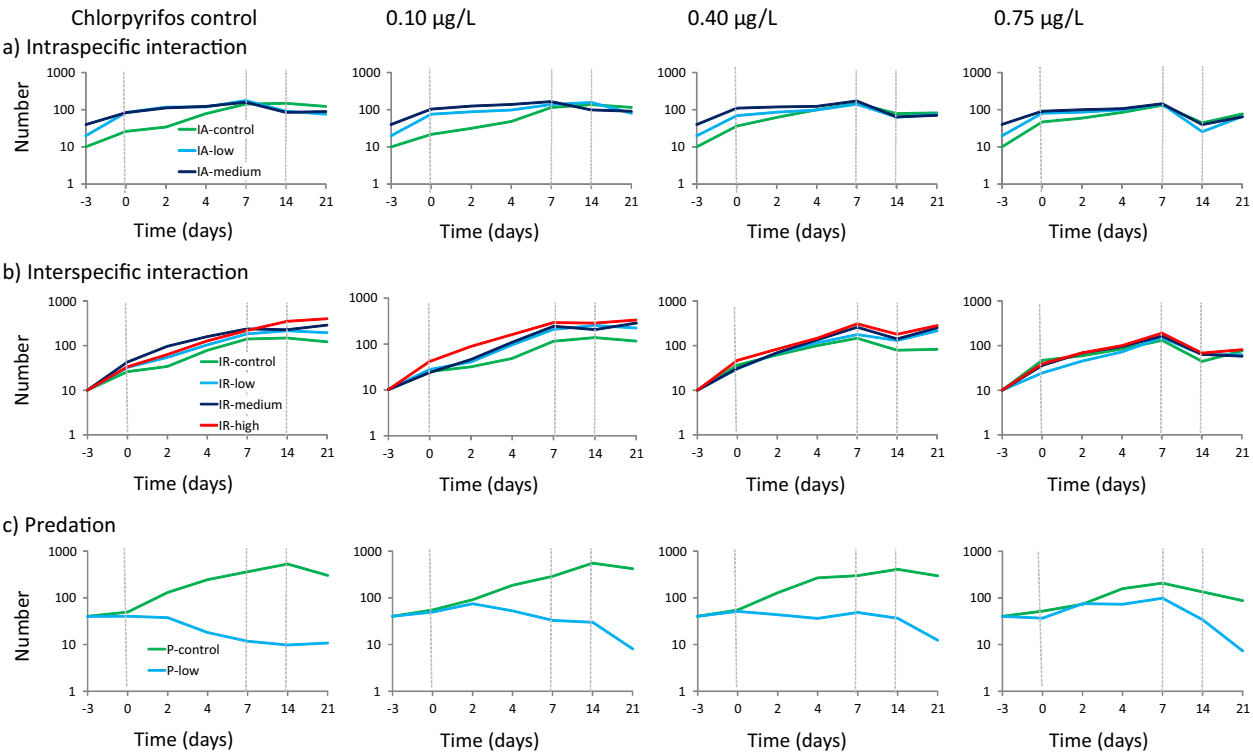

Figure 2. Total number of individuals in food-limited populations of *Daphnia pulex* under intraspecific interaction (**a**), interspecific interaction (**b**), and predation (**c**) for the different chlorpyrifos treatments (control, 0.10, 0.40, and 0.75 µg/L). Dotted lines indicate chlorpyrifos applications. IA = intraspecific; IR = interspecific.

number of juveniles was halved. This can be a result of the predator's feeding selectivity, mouth gape size, or feeding efficiency or the ability of prey to escape. Previous studies indicated that *Chaoborus* sp. are highly selective feeders and that feeding preferences can be dependent on their instar, which determines the mouth gape and, thus, size of consumable prey [15]. In addition, vulnerability of prey is the result of differing encounter rates, which are influenced by swimming speed of prey and the strike volume of the predator, where *Chaoborus* sp. has a high encounter rate with *D. pulex* compared with other organisms because of a large strike volume and a fast swimming speed [15]. If *Daphnia* swimming speed increases with body size [16] and encounter rate is a linear function of prey velocity, it is probable that the larger adults and subadults were faster and thus more likely to encounter an ambush predator than juveniles. As an example, Pastorok [17] showed that encounter rate, strike efficiency, and profitability from *Chaoborus trivittatus* reach a maximum when *Daphnids* are approximately 1.5 mm in length, supporting the hypothesis that juvenile *D. pulex* were most likely too small for *Chaoborus* sp. to effectively be consumed.

Combined effects of chlorpyrifos, intraspecific and interspecific interaction, and predation

In the present study, the combined effect of chlorpyrifos and intraspecific and interspecific interaction increased *G. pulex* survival to chlorpyrifos (Figure 1). Under intraspecific interaction, *G. pulex* survival was significantly greater 2 d after the first pulse until day 11 (Table 3). The calculated LC50 4-d values for *G. pulex* under intraspecific interaction were 0.14, 0.22, and 0.43 µg/L in the control, low, and medium intraspecific interaction treatments, respectively (Table 4). This trend of *G. pulex* survival increasing with density suggests that increased consumption through cannibalism and intraguild predation may be at play. For example, Dick [18] indicated the cannibalistic behavior of *Gammarus* sp. is regulated by food scarcity pressure. Chlorpyrifos-induced death of an individual in the high intraspecific treatment may have released the others from food scarcity, thereby increasing their resistance to chlorpyrifos stress.

Similarly, interspecific interaction resulted in significantly higher *G. pulex* survival, which began on day 2, continued for the duration of the experiment (Table 3), and yielded LC50 calculations for day 11 of 0.05, 0.14, and 0.13 µg/L in the low, medium, and high interspecific interaction treatments, respectively (Table 4). The LC50 4-d values could not be calculated for the high interaction treatments because of a lack of sufficient mortality. In the interspecific experiment it was clear that intraguild predation of *A. aquaticus* by *D. pulex* decreased as chlorpyrifos concentration increased (Supplemental Data, Figure S2). Similar interactive responses were observed for other pesticides by Blockwell et al. [19] and Del Arco et al. [3]. In the present study, predation could have allowed for release from food limitation at lower concentrations whereby *G. pulex* survival increased despite chlorpyrifos stress. This was also reflected by Gui and Grant [20], who found food availability to outweigh effects of a toxin in *Drosophila melanogaster*.

Density-related stressors other than competition alone, such as cannibalism and intraguild predation by *G. pulex*, may have played a role in the present experiment. Under laboratory and field conditions, Gammaridae have been observed to be cannibalistic, intraguild predators, and predators of other taxa, including *Asellus* sp. and fish [3,18,21].

Intraspecific interaction experiments with *D. pulex* showed the interaction of chlorpyrifos and intraspecific interaction to significantly increase total population size only on day 2 after

the start of the exposure period (Table 3). Subadults only significantly decreased after day 4 in the low and medium intraspecific treatments compared to the interaction controls (Supplemental Data, Table S2). Similar results have been reported by Viaene et al. [14] for *D. magna*, where intraspecific interaction reduced the effect of high pyrene exposure on population size. Interestingly, juvenile abundance initially decreased (days 0 and 2) and significantly increased on the last sampling day. This shows that the combined effect of chlorpyrifos and intraspecific interaction acts differently in different population age groups (i.e., with subadults and juveniles being more at risk) and that, although the total population size was not clearly affected, the combination of the 2 stressors can lead to effects on population demographics.

The interspecific interaction experiments with *D. pulex* showed that the interaction between interspecific interaction and chlorpyrifos had no significant effects on the total population size (Table 3). As explained earlier, evidence suggests that *D. pulex* could have been released from food limitation at lower chlorpyrifos concentrations by consuming their "competitor," *B. calyciflorus*. This is supported by the observed increase of *B. calyciflorus* abundance in test units in which *D. pulex* was not present, as well as in test units that contained both species and high chlorpyrifos concentrations (Supplemental Data, Figure S6). However, a different pattern was observed when comparing age classes. Interspecific interaction and chemical treatment moderately decreased subadult abundance on all days after day 4 (Supplemental Data, Table S2). Such an effect was more apparent at medium and high exposure concentrations (Supplemental Data, Figure S4). As observed in the intraspecific interaction test, subadults are more sensitive to the combined effects of interaction and chemical exposure. It is probable that only adult *D. pulex* (>0.8 mm) were large enough to consume *B. calyciflorus*, escaping food limitation, and that high juvenile abundances were just a result of the reproductive success of well-fitted adults.

The combination of chlorpyrifos and predation was found to significantly affect total population size in the first week of the exposure period (Table 3). *Daphnia pulex* abundance increased in treatments that contained *Chaoborus* sp. predation and chlorpyrifos compared with those only being affected by predation (Figure 2c). Previous experiments with *Chaoborus obscuripes* have shown chlorpyrifos EC50 4-d values of 0.18 μg/L in the laboratory [22] and 0.7 μg/L in the field [23] and 80% of immobilization after 0.7 μg/L exposure, which lasted for upward of 35 d [24]. This suggests that the chlorpyrifos concentrations used in the present experiment may have affected, to some extent, the predator's mobility and consumption efficiency. This was also reported by Viaene et al. [14], who explained that predation reduced the effect of high pyrene exposure through reductions in *Chaoborus* larvae feeding rates. The present results are in close agreement with Coors and De Meester [25], who found antagonistic responses in *D. magna* survival and other traits (e.g., size at maturity) using the insecticide carbaryl and the predator *Chaoborus crystallinus* as stressors.

In our predation experiment, significant interactive effects were also observed in population size (Supplemental Data, Table S2), with *D. pulex* juveniles and adults showing smaller differences with predation controls in the high exposure concentrations compared with those that had not received any chlorpyrifos exposure (Supplemental Data, Figure S5). It is possible that no interactive effects were seen in subadults because predation controls showed this age class to be most affected by chlorpyrifos. However, effects of chlorpyrifos were only seen after the second dosing, and combination effects were only seen prior to this point (Supplemental Data, Table S2). As indicated, it is likely that juveniles were not optimal prey for *Chaoborus* sp. [17]. Although this was not directly studied for chlorpyrifos, a reduced swimming speed as a result of chlorpyrifos exposure may have occurred in *D. pulex*, and larger individuals may have been consumed more frequently at low concentrations because the larger, slowed individuals were unable to escape as quickly as juveniles. Thus, these differences in age structure are most likely the result of a combination of optimal prey size, prey activity, and predator activity, the 3 possibly being affected by chemical stress. The present results contrast with those observed by Beketov and Liess [26], who indicated that the combination of predation and short-term pesticide exposure can lead to exhausted population regulation mechanisms of the brine shrimp *Artemia* spp. and to population size declines reaching extinction. In their experiment, however, predation was artificially simulated by extracting individuals from the experimental systems. The present study suggests that predator–prey relationships should be evaluated using both species at environmentally realistic ratios because pesticide exposure to predators can significantly influence the biological interaction with the prey and, therefore, can result in different population-level responses at different exposure levels.

CONCLUSION

The present study shows that stress caused by ecological interactions does not always result in a reduction of freshwater population size when exposed to a pesticide but may affect at least temporarily the demography of aquatic populations. However, species interactions and food habits could be affected in the presence of pesticide exposure, possibly leading to compensatory mechanisms that alleviate food limitation such as (intraguild) predation or cannibalism. Such responses are likely to be species-specific and context-specific and may be precursors of indirect effects leading to long-term impacts on community structure. The present data can be used to better understand complex species interactions and mechanisms that should be taken into account in chemical risk assessment. Chemical stress and species interactions should be further studied, making use of ecological models that allow the implementation of different ecological scenarios and extrapolation of individual-level or population-level responses to higher levels of biological organization.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3788.

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Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (paul.vandenbrink@wur.nl).

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