Supporting Information

Shrinking body size and physiology contribute to geographic variation and the higher toxicity of pesticides in a warming world

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S1 contains detailed information about the study populations and the associated thermal regimes during the experiment.

S2 contains detailed information on measured chlorpyrifos concentrations and water quality.

S3 contains detailed protocols of the physiological assays.

S4 contains detailed material and methods, and results of the independent action model for mortality.

S5 contains extended results.

S6 contains extended discussion about general effects of the pesticide.

The supporting Information contains eleven figures (S1-S11), eight tables (S1-S10), and is in total 45 pages.

Appendix S1. Details about the study populations and the associated thermal regimes during the experiment

Study populations

We studied three populations in Northern Europe (high latitude) and three populations in Southern Europe (low latitude). The populations at the high latitude came from southern Sweden: Hovgardsdammarna (57°14'24.3"N, 12°08'28.2"E) and Kalmar Dämme (56°40'04.6"N; 16°17'46.5"E), and from Denmark: Roskilde (55°39'09.8"N; 12°08'01.7"E). The populations at the low latitude came from southern France: Bassin de Réaltor (43°28'11.1"N; 05°19'44.1"E), La Durance (43°43'52.5"N; 05°44'53.0"E) and St. Martin de Crau (43°37'57.8"N; 04°46'55.1"E).

About 30 mated females were caught per population. They were immediately placed individually in plastic cups (7.5 cm height, 3.5 cm diameter) and provided with wet filter paper for oviposition. Filter papers containing eggs were transferred to the laboratory (KU Leuven, Belgium) where the eggs were kept at a water temperature of 22 °C and a 14h:10h light-dark cycle. Hatchlings were daily fed *Artemia* nauplii ad libitum. Ten days after the eggs hatched, larvae were allocated individually to plastic cups (5 cm height, 6 cm diameter) filled with 90 mL dechlorinated tap water. From this day onwards, larvae were fed *Artemia* nauplii ad libitum during weekdays (Monday-Friday).

Thermal regimes

The thermal regimes were based on the current mean water temperature and the current maximum DTF during the summer period in shallow (< 1m) freshwater ponds inhabited by the study species at both latitudes. At the high-latitude sites there is a mean summer water temperature of 20 °C with a maximum DTF of 5 °C (temperatures fluctuate daily between 17.5 °C and 22.5 °C). At the low-latitude sites the mean summer water temperature is 24 °C with a

maximum DTF of 10 °C (temperatures fluctuate daily between 19 °C and 29 °C)¹⁻³. Notably, the current thermal regime during summer in shallow ponds at the low latitude (mean of 24 °C and maximum DTF of 10 °C) corresponds to the future thermal regime predicted at the high latitude in 2100 under global warming scenario RCP 8.5 (IPCC 2013). This scenario indeed predicts an increase in mean temperature of 4 °C and an increase in maximum DTF of 5 °C by 2100^{4,5}. The maximum DTF at the high latitude by 2100 was estimated based on the daily maximum and minimum temperatures predicted by BCC_CSL 1.1 (Beijing Climate Center Climate System Model 1.1) under the RCP 8.5 scenario (following⁶). Further details on this method can be found in Verheyen and Stoks³.

The current water temperature data were obtained via the Flake model⁷. These data were validated by actual temperature data measured during summer in shallow waterbodies inhabited by the study species at both latitudes^{1,2}. Temperatures were measured by Hobo onset data loggers (TidbiT v2 Temp logger) placed at a depth of 30 cm. These loggers were also used during the laboratory experiment to collect water temperatures every 10 minutes in each thermal regime. The actual thermal regimes based on the measured water temperatures are shown in figure S1.

Figure S1. Data of measured water temperatures over a period of 24 hours are given for the six different thermal regimes: 3 levels of daily temperature fluctuations (constant = 0 °C, low = 5 °C and high = 10 °C) around a mean temperature of 20 °C (upper figure) and 24 °C (lower figure). Confidence intervals (95%) around the means of ca. 30 days are given by the grey area around the black lines.

Appendix S2: Information on measured chlorpyrifos concentrations and water quality

Details about the pesticide solutions

To obtain the CPF concentrations, a CPF stock solution of 1 mg/mL was made by dissolving CPF powder (Sigma-Aldrich, purity > 99%) in absolute ethanol (100%). The CPF stock solution was renewed monthly and stored in the dark in a cold (4°C) room. To acquire the targeted CPF concentrations, the stock solution was first diluted with Milli-Q water to obtain 0.75, 1.50 and 2.25 µg/mL and then with dechlorinated tap water to obtain the CPF exposure concentrations 0.75, 1.50 and 2.25 µg/L. In the solvent control the same amount of absolute (100%) ethanol was added (2.25 µL/L) as in the highest CPF concentration. This ethanol concentration is unlikely to cause effects since it is >5× lower than the NOEC for aquatic invertebrates⁸.

Details about the water samples

Water samples were taken to measure the CPF concentration at two time points: at the start and after 24 hours (just before daily renewal of the medium). At the start of the pesticide treatment, three water samples were taken per mean temperature (20°C and 24°C). After 24 hours, three water samples were taken at each of the six thermal regimes. UPLC MS/MS with Triple Quadrupole Mass Spectrometry was used to measure the CPF concentrations. Measured CPF concentrations are shown in Table S1. We also took water samples at the start (3 replicates per mean temperature) and after 24 hours of pesticide exposure (3 replicates per thermal regime) in all pesticide treatments (including the solvent control) to measure the following physico-chemical parameters: pH, hardness (mg/L CaCO₃), dissolved oxygen (mg/L) and conductivity (µS/cm). These results are shown in Table S2-S5.

Chlorpyrifos concentrations

Table S1. Data on measured chlorpyrifos (CPF) concentrations for each pesticide treatment: 0.75, 1.50 and 2.25 µg/L CPF.

Concentrations are given for both mean temperature treatments (20 °C and 24 °C) at the start of the pesticide treatment and after 24 hours just before renewal of the media. Per mean temperature three replicates were measured at the start, and after 24 hours three replicates were measured per DTF treatment at each mean temperature. Means and standard errors (SE) are given.

<u>Treatments:</u>			<u>0.75 µg/L CPF</u>		<u>1.50 µg/L CPF</u>		<u>2.25 µg/L CPF</u>	
Temperature	DTF	Time point	Mean	SE	Mean	SE	Mean	SE
20 °C	All DTF	Start	0.81	0.01	1.40	0.09	2.18	0.03
20 °C	0 °C DTF	After 24h	0.48	0.04	0.79	0.05	0.98	0.01
20 °C	5 °C DTF	After 24h	0.54	0.02	0.89	0.03	1.02	0.04
20 °C	10 °C DTF	After 24h	0.51	0.05	0.84	0.10	1.09	0.17
24 °C	All DTF	Start	0.81	0.01	1.45	0.13	2.22	0.07
24 °C	0 °C DTF	After 24h	0.44	0.01	0.69	0.16	1.02	0.01
24 °C	5 °C DTF	After 24h	0.50	0.04	0.79	0.07	1.13	0.11
24 °C	10 °C DTF	After 24h	0.58	0.03	0.81	0.05	1.21	0.03

Water quality parameters

Levels of all water quality parameters were very similar through time and across all thermal regimes and pesticide treatments (see Table S2-S5). Dissolved oxygen levels varied between 7.13 and 9.00 mg/ L. pH values varied between 8.15 and 8.31. Hardness levels varied between 158 and 165 mg/L CaCO₃. Conductivity levels varied between 600 and 611 μ S/cm.

Table S2. Data on water quality based on the following physico-chemical parameters: dissolved oxygen, pH, hardness and conductivity. Values of each physico-chemical parameter for both mean temperature treatments (20 °C and 24 °C) are given at the start of the control treatment (0 µg/L) and after 24 hours just before renewal of the media. Per mean temperature three replicates were measured at the start, and after 24 hours three replicates were measured per DTF treatment at each mean temperature. Means and standard errors (SE) are given.

Temperature	DTF	Time point	<u>Dissolved oxygen</u>		<u>pH</u>		<u>Hardness</u>		<u>Conductivity</u>	
			<u>(mg/L)</u>				<u>(mg/L CaCO₃)</u>		<u>(µS/cm)</u>	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
20 °C	All DTF	Start	8.97	0.05	8.26	0.02	160.00	0.00	599.67	0.33
20 °C	0 °C DTF	After 24h	7.84	0.11	8.15	0.03	164.67	0.67	603.67	0.67
20 °C	5 °C DTF	After 24h	8.05	0.12	8.27	0.04	162.00	1.15	610.33	2.91
20 °C	10 °C DTF	After 24h	8.49	0.09	8.23	0.09	162.00	0.00	608.33	1.20
24 °C	All DTF	Start	8.85	0.03	8.23	0.02	159.33	0.67	602.75	3.18
24 °C	0 °C DTF	After 24h	7.50	0.10	8.25	0.02	160.00	0.00	601.33	1.20
24 °C	5 °C DTF	After 24h	7.50	0.02	8.26	0.01	160.00	0.00	605.00	2.00
24 °C	10 °C DTF	After 24h	7.50	0.07	8.20	0.02	159.67	0.33	605.00	1.15

Table S3. Data on water quality based on the following physico-chemical parameters: dissolved oxygen, pH, hardness and conductivity. Values of each physico-chemical parameter for both mean temperature treatments (20 °C and 24 °C) are given at the start of the low pesticide treatment (0.75 µg/L) and after 24 hours just before renewal of the media. Per mean temperature three replicates were measured at the start, and after 24 hours three replicates were measured per DTF treatment at each mean temperature. Means and standard errors (SE) are given.

Temperature	DTF	Time point	<u>Dissolved oxygen</u>		<u>pH</u>		<u>Hardness</u>		<u>Conductivity</u>	
			<u>(mg/L)</u>				<u>(mg/L CaCO₃)</u>		<u>(µS/cm)</u>	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
20 °C	All DTF	Start	8.96	0.03	8.30	0.13	160.00	1.15	599.67	1.33
20 °C	0 °C DTF	After 24h	7.85	0.16	8.25	0.01	162.00	1.15	604.33	1.76
20 °C	5 °C DTF	After 24h	7.76	0.21	8.26	0.04	161.67	1.20	610.67	1.45
20 °C	10 °C DTF	After 24h	8.51	0.14	8.27	0.01	161.67	0.88	609.67	1.76
24 °C	All DTF	Start	8.87	0.01	8.24	0.01	159.33	1.33	602.17	0.33
24 °C	0 °C DTF	After 24h	7.62	0.12	8.24	0.02	160.67	1.76	601.33	0.33
24 °C	5 °C DTF	After 24h	7.62	0.06	8.31	0.03	160.00	1.45	604.67	1.76
24 °C	10 °C DTF	After 24h	8.05	0.03	8.21	0.02	158.00	1.76	606.00	1.53

Table S4. Data on water quality based on the following physico-chemical parameters: dissolved oxygen, pH, hardness and conductivity. Values of each physico-chemical parameter for both mean temperature treatments (20 °C and 24 °C) are given at the start of the medium pesticide treatment (1.5 µg/L) and after 24 hours just before renewal of the media. Per mean temperature three replicates were measured at the start, and after 24 hours three replicates were measured per DTF treatment at each mean temperature. Means and standard errors (SE) are given.

Temperature	DTF	Time point	<u>Dissolved oxygen</u>		<u>pH</u>		<u>Hardness</u>		<u>Conductivity</u>	
			<u>(mg/L)</u>				<u>(mg/L CaCO₃)</u>		<u>(µS/cm)</u>	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
20 °C	All DTF	Start	9.00	0.02	8.18	0.09	160.67	0.67	600.33	0.67
20 °C	0 °C DTF	After 24h	7.85	0.14	8.27	0.06	162.00	2.31	609.00	1.00
20 °C	5 °C DTF	After 24h	8.19	0.12	8.27	0.01	162.00	1.15	607.33	0.67
20 °C	10 °C DTF	After 24h	8.21	0.04	8.22	0.04	161.67	0.88	607.67	0.33
24 °C	All DTF	Start	8.99	0.01	8.26	0.02	158.00	1.16	602.50	0.33
24 °C	0 °C DTF	After 24h	7.31	0.10	8.25	0.02	160.00	0.00	600.67	1.20
24 °C	5 °C DTF	After 24h	7.49	0.03	8.26	0.03	158.67	0.67	603.00	1.00
24 °C	10 °C DTF	After 24h	7.81	0.12	8.22	0.02	158.67	0.67	609.00	3.06

Table S5. Data on water quality based on the following physico-chemical parameters: dissolved oxygen, pH, hardness and conductivity. Values of each physico-chemical parameter for both mean temperature treatments (20 °C and 24 °C) are given at the start of the high pesticide treatment (2.25 µg/L) and after 24 hours just before renewal of the media. Per mean temperature three replicates were measured at the start, and after 24 hours three replicates were measured per DTF treatment at each mean temperature. Means and standard errors (SE) are given.

Temperature	DTF	Time point	<u>Dissolved oxygen</u>		<u>pH</u>		<u>Hardness</u>		<u>Conductivity</u>	
			<u>(mg/L)</u>				<u>(mg/L CaCO₃)</u>		<u>(µS/cm)</u>	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
20 °C	All DTF	Start	8.95	0.02	8.25	0.05	160.67	0.67	602.00	2.08
20 °C	0 °C DTF	After 24h	7.69	0.13	8.27	0.01	161.33	0.67	607.00	2.08
20 °C	5 °C DTF	After 24h	8.06	0.09	8.26	0.02	161.33	0.67	606.33	0.33
20 °C	10 °C DTF	After 24h	7.86	0.03	8.24	0.01	160.67	0.67	605.00	1.15
24 °C	All DTF	Start	8.95	0.03	8.25	0.02	160.00	0.00	602.67	0.33
24 °C	0 °C DTF	After 24h	7.13	0.06	8.29	0.03	160.67	0.67	602.67	0.88
24 °C	5 °C DTF	After 24h	7.51	0.02	8.24	0.01	160.67	0.67	604.00	1.00
24 °C	10 °C DTF	After 24h	7.78	0.05	8.21	0.01	160.00	0.00	607.33	0.88

Appendix S3: Detailed protocols of the physiological assays

To spectrophotometrically quantify the different physiological traits, the head and the body of the larvae were first homogenized separately in PBS buffer (Phosphoric Buffered saline, for the head: 10% of the final mass x 10 μ L; for the body: 90% of the final mass x 10 μ L). Both homogenates were centrifuged at 13,000 g and 4 °C for 8 minutes to obtain the head and body supernatants. All physiological traits were measured on the body supernatant, except for the acetylcholinesterase (AChE) activity which was measured on the head supernatant. Protein content was measured (following the Bradford⁹ method) in both the head and body supernatants of each larva to correct the enzyme activities. One unit of AChE activity represents 1 μ mol acetylcholine hydrolyzed per minute; AChE activity was expressed as units per mg head protein. One unit of CytP450 represents 1 nmol 7-hydroxycoumarin formed per minute; one unit GST represents 1 μ mol GS-DNB formed per minute. One unit SOD activity was defined as the amount of SOD needed for a 50% inhibition of the WST-1 reduction; one unit CAT was defined as the amount of enzyme needed to degrade 1 nmol hydrogen peroxide per min. Activities of CytP450, GST, SOD and CAT were expressed as units per mg body protein. MDA concentrations were expressed as nmol MDA per mg fat.

The acetylcholinesterase (AChE) activity was quantified following the protocol of Campero et al.¹⁰. This enzyme is the target of the pesticide chlorpyrifos, which causes inhibition of AChE¹¹. Daily a reaction mixture of 255 μ L 10 mM acetylcholine iodide with 495 μ L 3 mM 5,5-dithiobis-2-nitro-benzoic acid (DTNB) was prepared. In each well of a 384-well microtiter plate 25 μ L of the reaction mixture was mixed with 5 μ L head supernatant. Thereafter, absorbance was measured at 412 nm every 30 sec for 30 minutes. The slopes of the linear part of the reaction curve of AChE were converted to enzyme units using the formula of Lambert-Beer.

One unit of AChE activity represents 1 μ mol acetylcholine hydrolyzed per minute. AChE activity was expressed as units per mg protein.

The cytochrome P450 monooxygenase (CytP450) activity was measured using a modified protocol based on the protocol of Anderson and Zhu¹². CytP450 is mainly responsible for the first phase in detoxifying xenobiotics in aquatic invertebrates¹³, including the pesticide chlorpyrifos¹⁴. During this first phase, the original compound may be oxidized into its more toxic metabolite¹⁵ by the binding of reactive or polar groups to the original compound¹⁶. Wells of a 96-well microtiter plate were filled in duplicate with 2 μ L body supernatant and 98 μ L 7-ethoxycoumarin (7-EC). This mixture was first incubated for 10 minutes at room temperature and thereafter, 100 μ L 2mM NADPH was added. Fluorescence was then measured every 30 sec at an excitation/emission wavelength of 390/465 nm for 20 minutes. The slopes of the linear part of the reaction curve CytP450 between 0 and 360 sec were converted in enzyme units using the law of Lambert-Beer. One unit of CytP450 activity represents 1 nmol 7-hydroxycoumarin formed per minute. CytP450 activity was expressed as units per mg protein.

The activity of glutathion-S-transferase (GST), an important phase 2 detoxification enzyme, was quantified following the protocol of McLoughlin et al.¹⁷. This enzyme is responsible for the conjugation of various xenobiotics having electrophilic centers (including organophosphates) with the substrate glutathione¹⁸. Daily, a substrate solution was made by mixing 33.33% 20mM glutathione (GSH) solution containing 1 mM ethylenediaminetetraacetic, 6.00% 0.02 mM phosphate buffer (pH 6.5) and 6.67% 40 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution. Wells of a 96-well UV microtiter plate were filled in duplicate with 5 μ L body supernatant and 195 μ L substrate solution. After a three-minute incubation at 30 °C, absorbance was measured at the same temperature (30 °C) every 30 sec at 340 nm for 6 minutes. The slopes

of the linear part of the reaction curve of GST were transformed into enzyme units by applying the formula of Lambert-Beer. One unit of GST activity is defined as 1 μmol GS-DNB formed per minute. GST activity was expressed as units per mg protein.

To measure antioxidant defense, activity levels of two important anti-oxidant enzymes in insects¹⁹ were quantified: superoxide dismutase (SOD) and catalase (CAT). Superoxide anions are first reduced to hydrogen peroxide by SOD and further to water by CAT. We used the SOD assay kit WST (Fluka, Buchs, Austria) and the protocol of De Block and Stoks²⁰ to measure SOD activity. In wells of a 96-well microtiter plate we added 20 μL body supernatant, 2 μL enzyme working solution and 40 μL WST working solution. After an incubation period of 20 minutes at 37 °C, formazan is formed by the reduction of tetrazolium salt WST-1 with superoxide anions. The absorbance of formazan was measured at 440 nm. SOD activity units are based on inhibition rates that were derived from an inhibition curve of commercial SOD made from bovine erythrocytes (> 97% purity). The amount of SOD needed for a 50% inhibition of the WST-1 reduction is defined as one unit SOD. CAT activity was measured following a protocol for damselfly larvae^{20,21}. In a first step, the body supernatant was diluted by a factor 16 (75 μL PBS buffer was added to 5 μL supernatant). In a second step, wells of a 96-well microtiter plate were filled with 20 μL diluted body supernatant, 80 μL PBS buffer and 100 μL of 20 mM hydrogen peroxide. The degradation of hydrogen peroxide to water was measured in duplicate at 240 nm every 15 sec for 2.5 minutes. The slope of the linear part of the absorbance curve was transformed into enzyme units by using the law of Lambert-Beer. One CAT activity unit is defined as the amount of enzyme needed to degrade 1 nmol hydrogen peroxide per min. Activity levels of SOD and CAT were expressed as units per mg protein.

As an estimate for oxidative damage to lipids (lipid peroxidation), we quantified the level of malondialdehyde (MDA)²². MDA levels were measured using the thiobarbituric acid (TBA) assay derived from a protocol of Miyamoto et al.²³. First, 50 μ L body supernatant was mixed with 50 μ L TBA solution (0.4 %, in 0.1 M HCl), which was then incubated at 90 °C for 60 minutes. After adding 165 μ L butanol and mixing the solutions, the mixtures was centrifuged at 845 g for 4 minutes. Finally, wells of a 384-well microtiter plate were filled in triplicate with 30 μ L of the mixture and fluorescence was measured at an excitation/emission wavelength of 535/550 nm. The standard curve of 1,1,3,3-tetramethoxypropan 99% malonaldehyde bis (dimethyl acetol) 99% was used to calculate MDA concentrations. MDA concentrations were expressed as nmol MDA per mg fat.

Total fat content was quantified based on a protocol of Marsh and Weinstein²⁴, which was modified for damselfly larvae²⁵. After filling glass tubes with 8 μ L body supernatant and 56 μ L sulphuric acid (100%), the tubes were heated for 20 minutes at 150 °C. After the tubes cooled down, 64 μ L Milli-Q water was added. Wells of a 380-well microtiter plate were filled in triplicate with 30 μ L of the final mixture. Absorbance was measured at 490 nm and a glyceryl tripalmitate standard curve was used to convert the measured absorbance into total fat content. Total fat content was used to correct the MDA concentrations.

Appendix S4. Detailed material and methods, and results of the independent action model for mortality

Material and methods

An interaction between stressors is antagonistic when its combined effect is smaller than the predicted effect by the null model, while it is synergistic when its combined effect is bigger than the predicted effect by the null model^{26,27}. To test for such deviations from the null model for mortality we used the Independent action (IA) model as recommended by Schäfer and Piggott²⁶. We applied the IA model separately for the two-way interactions Mean T × Pesticide (Table S6A) and DTF × Pesticide (Table S6B), and the three-way interaction Mean T × DTF × Pesticide (Table S6C). For the Mean T × Pesticide interaction, we tested at each chlorpyrifos concentration whether the higher mean temperature (24 °C) made chlorpyrifos more toxic compared to exposure at the mean temperature of 20 °C. For the DTF × Pesticide interaction we tested for each combination of DTF (5 °C and 10 °C) and chlorpyrifos concentration whether DTF made the pesticide more toxic compared to exposure at 0 °C DTF. For the Mean T × DTF × Pesticide interaction, we tested for each combination of a higher mean temperature (24 °C), DTF (5 °C and 10 °C) and chlorpyrifos concentration whether the combination of a higher mean temperature and DTF made the pesticide more toxic compared to exposure at 20 °C with 0 °C DTF.

The IA model was applied following the procedure of Coors and De Meester²⁸. Before calculating the predicted joint effects, the observed absolute effects of each single stressor needed to be converted to proportional effects. We therefore used the equation

$$E_i = \frac{(e_i - e_{control})}{(e_{max} - e_{control})},$$
 whereby the single-stressor effect (e_i) and the effect of the stressor-free control ($e_{control}$) are both in absolute units, while the maximum possible effect of that single

stressor ($e_{\max}$) for mortality is 100. To calculate the predicted joint effects, the following equation was used: $E_{\text{joint}} = 1 - \prod^i (1 - E_i)$. To make comparisons with the observed joint effects, the predicted joint effect E_{joint} needed to be back-transformed to absolute units by using the following equation: $e_{\text{joint}} = E_{\text{joint}} \times (e_{\max} - e_{\text{control}}) - e_{\text{control}}$. The 95% confidence interval of the observed effect (in absolute units) was used to determine if the observed effect significantly differed from the joint effect predicted by the model. The interaction is considered non-additive by the IA model when the predictive effect was not in the 95% confidence interval of the observed effect²⁸.

Results

The IA model indicated that a higher mean temperature synergistically increased the chlorpyrifos-induced mortality at all chlorpyrifos concentrations (Table S6A). The IA model also identified that the interaction between 5°C DTF and chlorpyrifos was additive at low and medium chlorpyrifos, while it was synergistic at high chlorpyrifos (Table S6B). The interaction between 10°C DTF and chlorpyrifos was at each chlorpyrifos concentration synergistic.

When considering the three-way interaction, the IA model identified that the interaction between a higher mean temperature, 5°C DTF and chlorpyrifos was also additive at low and medium chlorpyrifos, while it was synergistic at high chlorpyrifos (Table S6C). Furthermore, this IA model also indicated that the combination of a higher mean temperature and 10°C DTF synergistically increased the chlorpyrifos-induced mortality at all chlorpyrifos concentrations (Table S6C). Thus, the strongest increase in chlorpyrifos-induced mortality was at the combination of a high mean temperature and a high DTF; this synergistic interaction was present at all chlorpyrifos concentrations but strongest at the high chlorpyrifos concentration (difference

between predicted value of model and lowest value of observed 95% confidence interval is 41 %, other values < 20 %).

Table S6. Results of the independent action models identifying the interaction type for the response variable mortality between (A) mean temperature (Mean T) and the pesticide chlorpyrifos (CPF), (B) Mean T and CPF, and (C) Mean T, DTF and CPF.

(A) Two-way interaction: Mean T × Pesticide						
Mean T	Pesticide	Predicted	Observed	95% CI observed	Interaction effect	
24°C	Low CPF	1.86	11.46	[6.94, 15.98]	Synergistic	
24°C	Medium CPF	18.72	29.76	[24.11, 35.42]	Synergistic	
24°C	High CPF	28.83	51.78	[46.65, 56.91]	Synergistic	
(B) Two-way interaction: DTF × Pesticide						
DTF	Pesticide	Predicted	Observed	95% CI observed	Interaction effect	
5°C	Low CPF	6.03	7.38	[2.72, 12.03]	Additive	
5°C	Medium CPF	22.73	22.00	[15.35, 28.65]	Additive	
5°C	High CPF	23.78	40.00	[33.19, 46.81]	Synergistic	
10°C	Low CPF	4.34	14.71	[8.73, 20.68]	Synergistic	
10°C	Medium CPF	21.35	33.33	[26.70, 39.97]	Synergistic	
10°C	High CPF	22.42	57.30	[51.50, 63.09]	Synergistic	
(C) Three-way interaction: Mean T × DTF × Pesticide						
Mean T	DTF	Pesticide	Predicted	Observed	95% CI observed	Interaction effect
24°C	5°C	Low CPF	3.48	9.52	[2.22, 16.83]	Additive
24°C	5°C	Medium CPF	14.08	18.92	[9.93, 27.90]	Additive
24°C	5°C	High CPF	19.81	48.70	[39.52, 57.87]	Synergistic
24°C	10°C	Low CPF	1.70	20.00	[10.56, 29.44]	Synergistic
24°C	10°C	Medium CPF	12.5	39.81	[30.31, 49.31]	Synergistic
24°C	10°C	High CPF	18.34	66.47	[59.42, 73.53]	Synergistic

Appendix S5. Extended results

Results

Mortality

Exposure to 24 °C increased mortality compared to 20 °C (main Mean T effect, Table S8, Fig. S2), but only in high-latitude larvae (Mean T $\times$ Latitude, Table S8, Fig. S2; contrasts: $P < 0.001$; other $P = 0.47$).

Growth rate

Growth rate was lower at 24 °C compared to 20 °C (main Mean T effect, Table S7), and growth rate tended to decrease at 5 °C DTF (contrast: $P = 0.051$) and did decrease at 10 °C DTF (main DTF effect, Table S7; contrast: $P < 0.001$). However, the DTF effect depended on latitude (DTF $\times$ Latitude, Table S7, Fig S3), which further depended on mean temperature (Mean T $\times$ DTF $\times$ Latitude, Table S7, Fig S3). Only low-latitude larvae had lower growth rates with increasing DTF levels, which was stronger at the mean of 20 °C and already occurring at 5 °C DTF (contrast: $P < 0.001$), while at 24 °C the decrease in growth rate only occurred at 10 °C DTF (contrast: $P < 0.001$).

AChE activity

Activity levels of AChE were higher at 24 °C compared to 20 °C (main Mean T effect) and higher in low-latitude compared to high-latitude larvae (main Latitude effect) (Table S8, Fig. S4).

Detoxification enzymes (Cyt P450 and GST)

Exposure to 10 °C DTF increased CytP450 activity but only in low-latitude larvae at 20 °C (Mean T $\times$ DTF $\times$ Latitude, Table S8, Fig. S5; contrasts: $P < 0.001$, other $P > 0.11$). Low-latitude larvae had a higher CytP450 activity than high-latitude larvae (main Latitude effect), but

only at 20 °C combined with 10 °C DTF (Mean T $\times$ DTF $\times$ Latitude, Table S8, Fig. S5; contrasts: $P = 0.017$, other $P > 0.14$).

GST activity was lower at 24 °C compared to 20 °C (main Mean T effect, Table S8) and lower at 10 °C DTF compared to 0 °C DTF in low-latitude larvae (DTF $\times$ Latitude, Table S8, Fig. S6; contrasts: $P < 0.001$, other $P > 0.26$). Low-latitude larvae had a higher GST activity, but only at 0 and 5 °C DTF (DTF $\times$ Latitude; contrasts: both $P < 0.002$, other $P = 0.67$).

Oxidative stress and damage

In general, low-latitude larvae had a higher SOD activity than high-latitude larvae (main Latitude effect, Table S8, Fig. S7).

Low-latitude larvae had a higher CAT activity compared to high-latitude larvae at 0 °C (contrast: $P = 0.002$) and 5 °C ($P = 0.024$) DTF (DTF $\times$ Latitude, Table S8, Fig. S8).

Furthermore, both 5 °C and 10 °C DTF caused a decrease in CAT activity compared to 0 °C DTF in low-latitude larvae (DTF $\times$ Latitude, Table S8, contrasts: both $P < 0.050$; for high-latitude larvae, both $P > 0.41$), and at the mean of 20 °C (Mean T $\times$ DTF, Table S8, contrasts: both $P < 0.043$; for 24 °C, both $P > 0.59$).

Exposure to increasing DTFs caused higher MDA levels but only in low-latitude larvae and especially at 20 °C (Mean T $\times$ DTF $\times$ Latitude, Table S8, Fig. S9; contrasts: both $P < 0.034$; for high-latitude larvae: all $P > 0.37$). At 24 °C, only 10 °C DTF tended to increase MDA levels in low-latitude larvae (contrast: $P = 0.071$, other $P = 0.22$; for high-latitude larvae: all $P > 0.32$).

1 **Tables**

2 **Table S7.** Results of linear mixed models testing for the effects of mean temperature (Mean T), daily temperature fluctuation (DTF),
3 and latitude (Lat) on the total surface area; and of Mean T, DTF, Lat and pesticide (Pest) on larval growth rate in final instar *Ischnura*
4 *elegans* damselfly larvae. For growth rate, we ran besides a basic model also an extended model that included total surface area and its
5 interaction with pesticide. Bold *P*-values are significant (< 0.05).

Effect	Total surface area			Growth rate		Growth rate	
	df	χ^2	<i>P</i>	(basic model)		(extended model)	
				χ^2	<i>P</i>	χ^2	<i>P</i>
Mean T	1	67.80	<0.001	106.95	<0.001	85.83	<0.001
DTF	2	248.21	<0.001	58.31	<0.001	27.62	<0.001
Pesticide	3	/	/	43.26	<0.001	22.29	<0.001
Lat	1	2.60	0.11	0.55	0.46	0.34	0.56
Mean T × DTF	2	2.32	0.31	2.55	0.28	3.33	0.19
Mean T × Pest	2	/	/	1.65	0.65	0.059	1.00
DTF × Pest	6	/	/	18.48	0.0051	6.86	0.33
Mean T × Lat	1	50.91	<0.001	0.45	0.50	2.88	0.090

DTF × Lat	2	28.11	<0.001	22.36	<0.001	16.65	<0.001
Pest × Lat	3	/	/	3.76	0.29	5.87	0.12
Mean T × DTF × Pest	6	/	/	4.66	0.59	4.90	0.56
Mean T × DTF × Lat	2	1.22	0.54	8.94	0.011	7.29	0.026
Mean T × Pest × Lat	3	/	/	0.93	0.82	0.35	0.95
DTF × Pest × Lat	6	/	/	6.78	0.34	4.89	0.56
Mean T × DTF × Pest × Lat	6	/	/	4.52	0.61	3.89	0.69
Total surface area	1	/	/	/	/	26.53	<0.001
Pest × Total surface area	3	/	/	/	/	19.08	<0.001

7 **Table S8.** Results of bias-reduced generalized linear models testing for the effects of mean temperature (Mean T), daily temperature
8 fluctuation (DTF), pesticide (Pest), and latitude (Lat) on mortality in final instar *Ischnura elegans* damselfly larvae. We analyzed the
9 effects on two subsets: (left) for 0 and 0.75 µg/L chlorpyrifos (CPF), and (right) for 1.50 and 2.25 µg/L CPF. For each subset we ran
10 besides a basic model also an extended model that included total surface area and its interaction with pesticide. Bold *P*-values are
11 significant (< 0.05). All 3- and 4-way interactions that were not significant (*P* > 0.10), were removed from the models and are not
12 reported.

Effect	df	<u>0 and 0.75 µg/L CPF</u>				<u>1.50 and 2.25 µg/L CPF</u>			
		Basic model		Extended model		Basic model		Extended model	
		χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Mean T	1	0.31	0.58	0.17	0.68	29.03	<0.001	49.55	<0.001
DTF	2	9.29	0.010	5.18	0.075	55.82	<0.001	25.64	<0.001
Pest	1	10.96	<0.001	4.74	0.029	29.46	<0.001	37.37	<0.001
Lat	1	12.48	<0.001	20.93	<0.001	2.41	0.12	61.01	<0.001
Mean T × DTF	2	1.49	0.48	1.97	0.37	2.20	0.33	4.98	0.083
Mean T × Pest	1	8.69	0.003	9.75	0.002	2.32	0.13	2.21	0.14
DTF × Pest	2	1.75	0.42	0.00	1.00	6.63	0.036	14.72	<0.001

Mean T × Lat	1	0.59	0.44	1.15	0.28	3.99	0.046	3.48	0.062
DTF × Lat	2	3.88	0.14	0.85	0.65	6.28	0.043	2.36	0.31
Pest × Lat	1	6.17	0.013	2.31	0.13	0.38	0.54	2.60	0.11
Mean T × DTF × Pest	2	/	/	/	/	5.63	0.060	5.78	0.056
Total surface area	1	/	/	22.13	<0.001	/	/	22.67	<0.001
Pest x Total surface area	1	/	/	6.77	0.009	/	/	2.39	0.12

Table S9. Results of linear mixed models testing for the effects of mean temperature (Mean T), daily temperature fluctuation (DTF), pesticide (Pest), and latitude (Lat) on activity levels of cytochrome-P450 (CytP450), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT), and on malondialdehyde (MDA) levels in final instar *Ischnura elegans* damselfly larvae. Bold *P*-values are significant (< 0.05).

Effect	df	AChE activity		CytP450 activity		GST activity		SOD activity		CAT activity		MDA levels	
		χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Mean T	1	8.08	0.0045	0.86	0.35	18.11	<0.001	2.82	0.093	1.54	0.22	0.0047	0.95
DTF	2	0.31	0.86	6.91	0.032	14.07	<0.001	1.23	0.54	16.71	<0.001	5.14	0.077
Pest	3	24.59	<0.001	13.39	0.0039	1.42	0.70	1.76	0.62	11.15	0.011	19.33	<0.001
Lat	1	39.30	<0.001	4.43	0.035	27.43	<0.001	19.87	<0.001	10.48	0.0012	17.42	<0.001
Mean T × DTF	2	3.92	0.14	11.77	0.0028	5.12	0.077	1.45	0.49	10.40	0.0055	4.63	0.099
Mean T × Pest	2	0.86	0.83	2.49	0.48	3.91	0.27	1.72	0.63	4.94	0.18	0.87	0.83
DTF × Pest	6	1.57	0.95	2.76	0.84	4.66	0.59	3.83	0.70	7.34	0.29	17.26	0.0084
Mean T × Lat	1	0.020	0.90	3.06	0.080	2.11	0.15	0.0013	0.97	0.14	0.71	60.69	<0.001
DTF × Lat	2	2.59	0.27	5.84	0.054	10.19	0.0061	0.92	0.63	14.13	<0.001	6.20	0.045
Pest × Lat	3	1.59	0.66	0.26	0.97	0.26	0.97	1.40	0.71	5.90	0.12	3.79	0.29

Mean T × DTF × Pest	6	1.38	0.07	5.02	0.54	4.04	0.67	7.27	0.30	8.99	0.17	4.12	0.66
Mean T × DTF × Lat	2	0.95	0.62	6.49	0.039	0.55	0.76	1.24	0.54	3.08	0.21	14.25	<0.001
Mean T × Pest × Lat	3	1.36	0.72	1.67	0.64	1.50	0.68	4.52	0.21	3.86	0.28	7.59	0.055
DTF × Pest × Lat	6	2.06	0.91	2.45	0.87	3.84	0.70	3.43	0.75	4.02	0.67	10.67	0.099
Mean T × DTF × Pest × Lat	6	6.81	0.34	5.20	0.52	0.30	1.00	1.28	0.97	4.53	0.61	10.15	0.12

Table S10. Overview of all effects of the pesticide chlorpyrifos (CPF) and how these were influenced by mean temperature (Mean T) and daily temperature fluctuation (DTF) for the measured response variables. The direction of the arrows indicates whether the CPF effect on that response variable was significantly strengthened (↑) or weakened (↓); the thickness of the arrows indicates the magnitude of the effect. When at a given thermal condition there was no significant effect of CPF (compared to the control treatment, hence 0 µg/L CPF) this is presented as ‘No effect’. When 5 °C or 10 °C DTF did not increase the CPF-induced mortality compared to 0 °C DTF at medium (1.50 µg/L) or high (2.25 µg/L) CPF, this is indicated as ‘/’.

Endpoint	Effects of chlorpyrifos (CPF)		
	Low (0.75 µg/L) CPF	Medium (1.50 µg/L) CPF	High (2.25 µg/L) CPF
Mortality	20 °C & all DTF: ↑ (+2.7 %) 24 °C & all DTF: ↑ (+5.5 %)	20 °C & 5 °C DTF: / (+11 %) 20 °C & 10 °C DTF: / (+10 %) 24 °C & 5 °C DTF: / (−7 %) 24 °C & 10 °C DTF: ↑ (+15 %)	20 °C & 5 °C DTF: / (+8 %) 20 °C & 10 °C DTF: ↑ (+22 %) 24 °C & 5 °C DTF: ↑ (+25 %) 24 °C & 10 °C DTF: ↑ (+44 %)
	Smaller larvae were more sensitive to CPF than bigger larvae (↑ mortality)		
Growth rate	0 °C DTF: No effect (−11 %) 5 °C DTF: ↓ trend (−19 %) 10 °C DTF: ↓ (−47 %)	0 °C DTF: No effect (−16 %) 5 °C DTF: ↓ trend (−18 %) 10 °C DTF: ↓ (−57 %)	0 °C DTF: ↓ trend (−17 %) 5 °C DTF: No effect (−10 %) 10 °C DTF: ↓ (−51 %)
	Smaller larvae were more sensitive to CPF than bigger larvae (↓ growth rate)		
AChE activity	Across thermal regimes: ↓	Across thermal regimes: ↓	Across thermal regimes: ↓
CytP450 activity	No effect	No effect	Across thermal regimes: ↑
GST activity	No effect	No effect	No effect
SOD activity	No effect	No effect	No effect
CAT activity	No effect	Across thermal regimes: ↓	Across thermal regimes: ↓
MDA levels	No effect	No effect	0 & 5 °C DTF: No effect 10 °C DTF: ↑

Figures

Figure S2. Mean (± 1 SE) mortality (%) of low-latitude and high-latitude *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and pesticide exposure.

Figure S3. Mean (± 1 SE) growth rate (day⁻¹) of low-latitude and high-latitude *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and pesticide exposure.

Figure S4. Mean (± 1 SE) acetylcholinesterase (AChE) activity of low-latitude and high-latitude *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and pesticide exposure.

Figure S5. Mean (± 1 SE) cytochrome P450 monooxygenase (CytP450) activity of low-latitude and high-latitude *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and pesticide exposure.

Figure S6. Mean (± 1 SE) glutathione-S-transferase (GST) activity of low-latitude and high-latitude *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and pesticide exposure.

Figure S7. Mean (± 1 SE) superoxide dismutase (SOD) activity of low-latitude and high-latitude *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and pesticide exposure.

Figure S8. Mean (± 1 SE) catalase (CAT) activity of low-latitude and high-latitude *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and pesticide exposure.

Figure S9. Mean (± 1 SE) malondialdehyde (MDA) levels of low-latitude and high-latitude *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and pesticide exposure.

Figure S10. Mean ($\pm$ 1 SE) acetylcholinesterase (AChE) activity (a), cytochrome P450 monooxygenase (CytP450) activity (b) and catalase (CAT) activity (c) of *Ischnura elegans* damselfly larvae as a function of pesticide exposure.

Figure S11. The correlation between total surface area and surface area to volume ratio.

Appendix S6. Extended discussion about general effects of the pesticide

General effects of the pesticide chlorpyrifos (CPF)

Exposure to CPF increased mortality and reduced growth rate of *I. elegans* damselfly larvae thereby confirming previous studies in the study species^{29–31}. CPF-induced growth reductions can be explained by an energy allocation away from growth towards maintenance³², respiration³³ and/or defense and repair mechanisms³⁴. Consistent with the latter explanation and other studies on invertebrates^{14,35}, larvae had higher levels of the first phase detoxification enzyme cytochrome P450 (CytP450) when exposed to the high CPF concentration. Furthermore, CPF inhibited its target enzyme acetylcholinesterase (AChE). This is a widespread response in freshwater insects¹¹, including the study species *I. elegans*^{29,30}. Acetylcholinesterase plays an important role in neurotransmission¹¹ and has been associated with a decreased food intake³⁶, which may also have contributed to the lowered growth rate under CPF exposure. Furthermore, the inhibition of AChE may cause mortality¹¹. The toxic effects on mortality and growth rate may also be explained by higher levels of oxidative stress and damage, since exposure to CPF can generate reactive oxygen species (ROS³⁷). Moreover, CPF reduced the antioxidant defense as indicated by reduced levels of catalase (CAT), one of the key antioxidant enzymes in insects¹⁹. The combination of higher production of ROS and an impaired antioxidant defense may explain why CPF-exposed larvae had higher levels of oxidative damage to lipids (measured as malondialdehyde, MDA levels). This finding is in line with previous studies^{37,38} where CPF exposure impaired antioxidant enzymes (e.g. CAT) and increased oxidative damage to lipids.

Abbreviations

AChE, acetylcholinesterase; BCC_CSL 1.1, Beijing Climate Center Climate System Model 1.1; CAT, catalase; CPF, chlorpyrifos; CytP450, cytochrome P450 monooxygenase; DTF, daily temperature fluctuation; GST, glutathione-S-transferase; IA, Independent Action; IPCC, Intergovernmental Panel on Climate Change; Lat, latitude; MDA, malondialdehyde; MeanT, mean temperature; NOEC, No Effect Concentration; Pest, pesticide; RCP 8.5, Representative Concentration Pathway 8.5; ROS, reactive oxygen species; SOD, superoxide dismutase; UPLC MS/MS, ultra-performance liquid chromatography - tandem mass spectrometer.

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