

1 **Increased daily temperature fluctuations overrule the ability of gradual thermal evolution to**
2 **offset the increased pesticide toxicity under global warming**

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10 **Keywords:** “Climate-induced toxicant sensitivity” (CITS) concept; Evolutionary ecotoxicology;

11 Geographic variation; Multiple stressors; “Toxicant-induced climate change sensitivity” (TICS)

12 concept; Upper thermal tolerance

13

14 **Abstract:**

15 The widespread evidence that global warming can increase species sensitivities to chemical
16 toxicants and vice versa, and the recent insight that thermal evolution may mitigate these effects is
17 crucial to predict the future impact of toxicants in a warming world. Nevertheless, a major
18 component of global warming, the predicted increase in daily temperature fluctuations (DTFs), has
19 been ignored at the interface of evolutionary ecotoxicology and global change biology. We studied
20 whether 4°C warming and an 5°C DTF increase (to 10°C DTF) magnified the negative impact of
21 the insecticide chlorpyrifos (CPF) in larvae of low- and high-latitude populations of the damselfly
22 *Ischnura elegans*. While 4°C warming only increased CPF-induced mortality in high-latitude
23 larvae, the high (10°C) DTF increased CPF-induced larval mortality at both latitudes. CPF reduced
24 the heat tolerance, however, this was buffered by latitude-specific thermal adaptation to both mean
25 temperature and DTF. Integrating our results in a space-for-time substitution indicated that gradual
26 thermal evolution in high-latitude larvae may offset the negative effects of CPF on heat tolerance
27 under warming, unless the expected DTF increase is taken into account. Our results highlight the
28 crucial importance of jointly integrating DTFs and thermal evolution to improve risk assessment
29 of toxicants under global warming.

30 **Introduction**

31 Chemical pollution and global warming are two major threats to organisms, that moreover can
32 interact¹⁻⁵. This constitutes a key challenge for a realistic ecological risk assessment under global
33 warming^{2,6,7}. Two concepts are pivotal at this intersection of ecotoxicology and global change
34 biology. On the one hand, higher mean temperatures can magnify the lethal effects of many
35 pesticides^{1,2} such as organophosphates (including the insecticide chlorpyrifos⁸⁻¹⁰). This is
36 encapsulated in the “climate-induced toxicant sensitivity” (CITS) concept^{2,4,11}. On the other hand,

37 many studies demonstrated that exposure to chemical toxicants can decrease the heat tolerance of
38 ectotherms¹²⁻¹⁸. The latter is captured by the “toxicant-induced climate change sensitivity” (TICS)
39 concept^{1,2,4}.

40 While there is increasing awareness that to better understand and predict the impact of
41 toxicants¹⁹ and of warming²⁰ an evolutionary perspective is pivotal, this has largely been ignored
42 in studies on the CITS and TICs concepts. Despite emerging insights that populations along
43 geographical gradients may differ both in toxicant sensitivity²¹ and in thermal sensitivity²⁰, studies
44 on the CITS and TICS concepts almost exclusively focused on single populations. This limits our
45 understanding of the role of thermal adaptation in shaping geographic variation in the strength of
46 both concepts. Moreover, this restricts our ability to predict the impact of gradual thermal
47 evolution in shaping the impact of toxicants under global warming. Indeed, in case of latitude-
48 associated thermal adaptation one can use the current sensitivity to high temperatures and to
49 chemical toxicants at high temperatures in the populations at warmer, lower latitudes to predict the
50 future sensitivity to warming and chemical toxicants under warming in high-latitude populations
51 (for example^{22,23}). This so-called space-for-time substitution approach thereby assumes gradual
52 thermal evolution of the high-latitude populations toward the phenotype of the low-latitude
53 populations^{20,24,25}. While powerful to predict the effects of micro-evolution²⁶, this approach has
54 never been used to address how gradual thermal evolution may shape the strength of the TICS and
55 CITS concepts under future warming.

56 Besides increases in mean temperature, increases in daily temperature fluctuations (DTFs)
57 are a largely overlooked key component of global warming²⁷. Recent ecological studies showed
58 that increases in DTFs may impact fitness more severely compared to increases in mean
59 temperature^{27,28}. Moreover, space-for-time substitution studies only take increases in mean
60 temperature into account and not increases in DTF. Recent evidence, however, showed that DTFs

61 may switch predictions of the impact of increases in mean temperature²⁹. DTFs have only recently
62 been integrated in ecotoxicology. Yet, the few studies all demonstrated DTF can increase chemical
63 toxicity (for pesticides³⁰⁻³²; for metals³³), highlighting the importance of integrating DTFs in the
64 CITS concept. This all indicates DTFs should be considered in studies at the interface of
65 evolutionary ecotoxicology and global change biology. Furthermore, the few studies so far
66 considered the effect of DTF on toxicity at a single mean temperature, yet the effects of DTFs on
67 fitness, hence likely also on sensitivity to chemical toxicants, may strongly differ in magnitude
68 depending on the mean temperature³⁴.

69 Although both the CITS and TICS concepts are necessary to understand and predict the
70 effects of chemical toxicants under global warming, they have been consistently studied in
71 isolation. Yet, it can be expected that both concepts are interconnected making studies of single
72 concepts potentially misleading. A notable exception is the study by Op de Beeck et al.¹⁸ showing
73 that exposure to an increase in mean temperature changed how the pesticide chlorpyrifos reduced
74 the heat tolerance, thereby illustrating the interdependence of both concepts. Studies how DTFs,
75 potentially in interaction with mean temperatures, may connect both concepts are missing.

76 We investigated the single and combined effects of increases in mean temperature and in
77 DTF on the sensitivity to the pesticide chlorpyrifos (CPF), and vice versa the effect of exposure to
78 CPF on the sensitivity to warming, both in low- and high-latitude populations of an aquatic insect.
79 This approach allowed to address in an integrated way the above identified knowledge gaps in the
80 TICS and CITS concepts: the influence of gradual evolution (thermal adaptation), the role of DTFs
81 and the interdependence of both concepts. Specifically, we tested: (i) if 4°C warming and a 5°C
82 increase in DTF increase CPF toxicity (CITS concept) by studying effects on two life-history traits
83 (mortality and growth rate); (ii) if exposure to CPF decreases heat tolerance (TICS concept); (iii) if
84 4°C warming and a 5°C DTF increase negatively impacted the CPF-induced decrease in heat

85 tolerance (interdependence of CITS and TICS); and (iv) if gradual thermal evolution can buffer for
86 the increased CPF toxicity under global warming (space-for-time substitution approach²⁵). The
87 organophosphate insecticide chlorpyrifos (CPF) is frequently applied worldwide³⁵, and is a top ten
88 chemical found in UK surface waters with the highest risk to aquatic wildlife³⁶. Moreover, it is
89 considered a priority pollutant by the European Water Framework Directive 2000/60/EC³⁷. As
90 study organisms we chose *Ischnura elegans* damselfly larvae which inhabit shallow freshwater
91 ponds that are subjected to large DTFs. Damselfly larvae are especially vulnerable to both
92 warming³⁸ and toxicants³⁹ since they cannot escape exposure to these stressors due to their
93 obligate aquatic life⁴⁰. We studied high-latitude (Denmark and southern Sweden) and low-
94 latitude (southern France) populations to assess the influence of thermal adaptation on the CITS
95 and TICS concepts and to assess the future strength of both concepts under global warming in the
96 high-latitude populations by applying a space-for-time substitution²⁵.

97 **Materials and methods**

98 *Study populations*

99 Early summer 2017 (mid of June – mid of July), 30 mated females of the damselfly *I. elegans*
100 were sampled at each of three populations in both the high-latitude region (Denmark and southern
101 Sweden) and the low-latitude region (southern France) of the species' European range⁴¹. All six
102 populations were located at shallow ponds; these were likely not directly exposed to pesticides
103 because they were situated >100 m from agricultural fields⁴², however, we cannot exclude the
104 possibility that they were exposed to very low pesticide concentrations. Given pesticide exposure
105 is in general higher at low latitudes than at high latitudes in Europe⁴³, it is possible that
106 immigration of damselflies from sites directly exposed to pesticides into the unexposed study
107 populations may have been larger at the low latitude. Yet, this was not mirrored in our results as
108 for example at 20°C larval mortality in low-latitude larvae was as high as in high-latitude larvae

109 (and even higher at 10°C DTF) (see results). The low-latitude populations were located in southern
110 France: St. Martin de Crau (43°37'57.8"N; 04°46'55.1"E), Bassin de Réaltor (43°28'11.1"N;
111 05°19'44.1"E) and La Durance (43°43'52.5"N; 05°44'53.0"E). The high-latitude populations
112 were located in southern Sweden: Hovgardsdammarna (57°14'24.3"N, 12°08'28.2"E) and Kalmar
113 Dämme (56°40'04.6"N; 16°17'46.5"E); and in Denmark: Roskilde (55°39'09.8"N;
114 12°08'01.7"E).

115 Mated females were placed individually in plastic cups (7.5 cm height, 3.5 cm diameter)
116 that contained wet filter papers for oviposition. Only filter papers that contained eggs were
117 transferred to the lab in Leuven (Belgium). After arrival, the eggs were immediately stored in a
118 temperature-controlled incubator with a constant water temperature of 22°C and a 14:10h light-
119 dark cycle. Ten day old hatchlings were separated and kept individually in plastic cups (5 cm
120 height, 6 cm diameter) containing 90 mL dechlorinated tap water. From this moment onwards,
121 larvae were fed *Artemia* nauplii ad libitum, five days per week (Monday-Friday).

122 *General experimental setup*

123 From hatching onwards, larvae were placed at one of six combinations of two mean temperatures
124 (20°C and 24°C) and three daily temperature fluctuation levels (DTFs: constant = 0°C, low = 5°C
125 and high = 10°C). One day after larvae had reached the final instar, they entered the 6-day
126 pesticide exposure period, resulting in 12 treatment combinations per latitude: 2 mean
127 temperatures × 3 DTFs × 2 pesticide treatments (solvent control and chlorpyrifos). Depending on
128 mortality, we started between 24 and 50 larvae per treatment combination (total of 852 larvae).
129 Larvae coming from the three populations per latitude were as equally divided as possible among
130 the different treatment combinations.

131 The temperature treatments were chosen based on (i) the current mean water temperature
132 and (ii) the current maximum DTFs during summer in shallow (<1m) freshwater ponds that the

133 study species inhabits at high latitude (mean of 20°C and maximum DTF of 5°C) and low latitude
134 (mean of 24°C and maximum DTF of 10°C). These data were derived from the Flake model⁴⁴ and
135 confirmed by temperature data measured by Hobo onset data loggers (TidbiT v2 Temp logger) in
136 both high- and low-latitude shallow ponds inhabited by the study species⁴⁵⁻⁴⁷. The same loggers
137 were used throughout the entire laboratory experiment to collect every 10 minutes water
138 temperature data in each temperature treatment. The collected water temperature data are shown
139 per temperature treatment in figure S1 (Appendix S1).

140 The current thermal conditions at the low latitude (mean summer water temperature of
141 24°C and maximum DTF of 10°C) correspond to the expected future thermal conditions at the
142 high latitude under global warming. This is according to the IPCC (2013)⁴⁸ Representative
143 Concentration Pathway (RCP) 8.5, which predicts by 2100 (i) a 4°C increase in mean temperature
144 and (ii) a 5°C increase in magnitude of DTF^{27,48}. The magnitude of DTF at high latitude was
145 estimated using daily maximum and minimum temperatures predicted by BCC_CSL 1.1 (Beijing
146 Climate Center Climate System Model 1.1) (following Paaijmans et al.⁴⁹). More details on this
147 method can be found in Verheyen and Stoks²⁹.

148 *Pesticide exposure*

149 When larvae moulted into the final instar they entered the 6-day pesticide exposure period. Larvae
150 were placed individually in glass jars (200 mL) that were filled with 100 mL medium (solvent
151 control or chlorpyrifos). All media were daily renewed (= static renewal). A chlorpyrifos (CPF)
152 concentration of 1 µg/L was selected based on a previous experiment where final instar larvae
153 were exposed to four concentrations (0, 0.75, 1.25 and 1.75 µg/L CPF) under the same
154 experimental conditions. At 1.25 µg/L CPF larvae suffered considerable mortality (ca. 45%) while
155 at 0.75 µg/L CPF there was only a slight growth reduction. In edge-to-field ponds where *I. elegans*
156 may be very abundant⁵⁰, CPF peak concentrations up to 100 µg/L can occur through run-off⁵¹.

157 The exposure concentration was obtained from a CPF stock solution (1 mg/mL) which was
158 stored in a dark room at 4°C and renewed monthly. To prepare this CPF stock solution, CPF
159 powder (Sigma-Aldrich, purity > 99%) was dissolved in ethanol (absolute, 100%). The stock
160 solution was then further diluted to acquire the targeted CPF concentration of 1 µg/L. A solvent
161 control was used with the same concentration (1 µL/L) of ethanol (absolute, 100%) as in the
162 pesticide treatment. This low ethanol concentration is assumed to have no effects on the response
163 variables since it is lower (12×) than the NOEC for aquatic invertebrates⁵² and much lower (66×)
164 than the recommended concentration of 100 µL/L⁵³.

165 Water samples were taken at two moments to measure the CPF concentrations in the glass
166 jars. At the start of the exposure period, water samples were taken from six jars per mean
167 temperature. After 24 hours (just before the medium was renewed), six water samples were taken
168 at each of the six thermal treatment combinations. CPF concentrations were measured using UPLC
169 MS/MS with Triple Quadrupole Mass Spectrometry at KU Leuven. The mean start CPF
170 concentration in the glass jars at the mean temperature of 20°C was 0.98 µg/L (SE: 0.04), while it
171 was 1.04 µg/L (SE: 0.04) at the mean temperature of 24°C. At the mean temperature of 20°C,
172 initial CPF concentrations decreased after 24 hours to 0.59 µg/L (SE: 0.03) at the constant (0°C)
173 DTF treatment, to 0.56 µg/L (SE: 0.05) at the low (5°C) DTF treatment and to 0.62 µg/L (SE:
174 0.05) at the high (10°C) DTF treatment. At the mean temperature of 24°C, initial CPF
175 concentrations decreased to 0.61 µg/L (SE: 0.05) at the constant (0°C), to 0.53 µg/L (SE: 0.06) at
176 the low (5°C) and to 0.63 µg/L (SE: 0.03) at the high (10°C) DTF treatment.

177 *Life-history traits*

178 Mortality was checked daily during the 6-day pesticide exposure period. To obtain growth rates,
179 each larva was weighed twice: at the start (initial mass) and at the end (final mass) of the 6-day

180 pesticide exposure period. Wet masses were obtained to the nearest 0.01 mg using an electronic
181 balance (Mettler Toledo® AB135-S, Columbus, Ohio, USA). Larval growth rates were calculated
182 as $[\ln(\text{final mass}) - \ln(\text{initial mass})] / 6$ days. Before the larvae were weighed, they were gently
183 blotted dry with tissue paper; this gives reliable wet mass data that strongly correlate with dry
184 mass⁵⁴. Sample sizes for larval growth rate varied between 23 and 37 larvae for each of the 12
185 treatment combinations (total of 730 larvae, see Fig. 2 for exact sample sizes).

186 *Heat tolerance: CTmax*

187 The heat tolerance of each larvae was assessed after the 6-day pesticide exposure period by
188 measuring the critical thermal maximum (CTmax). This is considered a good estimation to assess
189 an organism's vulnerability to warming⁵⁵. A dynamic method was used whereby larvae were
190 heated up at a constant rate until the temperature (CTmax) was reached where larvae neither
191 moved any body parts nor showed muscular spasms⁵⁶. For this, larvae were placed individually in
192 plastic vials (50 mL), which were kept floating in a temperature-controlled aquarium. Vials were
193 filled with dechlorinated tap water in the absence of the pesticide. The initial water temperature in
194 the vials was set at 20°C or 24°C depending on the mean water temperature of the larval treatment.
195 All CTmax tests started between 10h45 and 13h45, in which all DTF treatments reached the mean
196 temperature of either 20°C or 24°C.

197 We used a constant heating rate of 0.3°C per minute that was realized using an aquarium
198 heater (TC120 optima immersion thermostat, Cambridgeshire, UK). The applied heating rate lies
199 in the commonly used range when quantifying the CTmax of aquatic insects (for example^{56,57}).
200 Furthermore, this heating rate is considered to be fast enough to avoid acclimation and slow
201 enough for the larvae's body temperatures to closely track the raising water temperatures⁵⁷.

202 When larvae reached their CTmax, they were immediately transferred to their original
203 temperature treatment to recover. Only very few larvae (1.62%: 12 out of 742 larvae) did not

204 recover within 20 minutes after the CTmax test. These were presumed to be dead, and were
205 excluded from the CTmax analyses. The recovered larvae were weighed after the CTmax test. In
206 all trials the saturation of dissolved oxygen in the water of the vials remained above 80%. Water
207 temperatures were measured in a randomly chosen vial during each trial to check the heating rate
208 in each trial. Sample sizes for heat tolerance (CTmax) varied between 23 and 37 larvae for each of
209 the 12 treatment combinations (total of 730 larvae, see Fig. 3 for exact sample sizes).

210 *Statistical analyses*

211 The single and combined effects of mean temperature, DTF, pesticide exposure and latitude on
212 larval growth rate and heat tolerance were analyzed using linear mixed models within the ‘lme4’
213 package v1.1-18.1⁵⁸, and by using the ‘afex’ package v0.22-1⁵⁹. Effects on mortality (binary
214 response variable) were tested using a generalized linear mixed model with a binomial error
215 distribution and the logit-link function. Since almost no larval mortality occurred in the control
216 treatments (see results), the single and combined effects of mean temperature, DTF and latitude
217 were analyzed separately for larvae exposed to the solvent control and to the pesticide. This allows
218 more powerful tests whether the pesticide effect is stronger at the higher mean temperature and at
219 higher DTFs, and whether this differs between latitudes. Population nested in latitude was added in
220 each model as a random factor, although it was never significant. Furthermore, body mass was
221 added to the model as covariate in the analysis of heat tolerance. To meet ANOVA assumptions,
222 larval growth rates were square root transformed, while heat tolerances were boxcox transformed.

223 R 3.4.0. for Windows was used to statistically analyze the data⁶⁰. Wald chi-square statistics
224 and p-values for fixed effects were calculated by using the ‘car’ package v3.0-2⁶¹. Interactions
225 between treatment combinations were further analyzed by comparing least-square means using
226 Tukey posthoc tests obtained with the ‘lsmeans’ package v2.27-62⁶². Given the focus on the
227 effects of the pesticide and DTF, we interpreted interactions from this perspective.

228 **Results**

229 *Mortality*

230 The main model indicated that exposure to CPF caused a general increase in mortality (main effect
231 Pesticide: $\chi_2^2 = 23.50$, $P < 0.001$). Separate analyses per pesticide treatment showed that mortality
232 was low (on average 3.12%) in all solvent control treatments (Fig. 1). Neither mean temperature,
233 DTF or latitude, nor their interactions affected mortality in the absence of the pesticide (all $P >$
234 0.69 , Fig. 1). However, in pesticide-exposed larvae the 10°C DTF treatment caused in general an
235 increase in CPF-induced mortality of ca. 11% compared to the 0°C and 5°C DTF treatments (DTF:
236 $\chi_2^2 = 6.44$, $P = 0.040$). Furthermore, mean temperature and latitude interacted in pesticide-exposed
237 larvae (MeanT \times Latitude: $\chi_1^2 = 5.78$, $P = 0.016$, Fig. 1): CPF-induced mortality increased with ca.
238 19.4 % at 24°C compared to 20°C, but only in high-latitude larvae (Tukey: $P = 0.0037$).

239 *Growth rate*

240 Exposure to the pesticide reduced the growth rate (main effect Pesticide), yet only in low-latitude
241 larvae at 24°C (Tukey: $P < 0.001$) resulting in a significant Pest \times MeanT \times Lat interaction (Table
242 1, Fig. 2). In general, low-latitude larvae grew faster than high-latitude larvae (Latitude: Table 1,
243 Fig. 2). The effect of DTF depended on the mean temperature and latitude (DTF \times MeanT and
244 DTF \times MeanT \times Lat: Table 1, Fig. 2). At a mean of 20°C, growth rates at both latitudes were not
245 affected by DTF (Tukey: all $P > 0.37$). At a mean of 24°C, growth rate in high-latitude larvae
246 decreased at 5°C (Tukey: $P = 0.0010$) and 10°C DTF (Tukey: $P = 0.0057$) compared to 0°C DTF;
247 while growth rate in low-latitude larvae did not decrease when temperatures fluctuated (Tukey: all
248 $P > 0.27$).

249 *Heat tolerance (CTmax)*

250 Overall, larvae had a higher heat tolerance when reared at 24°C than at 20°C (main effect MeanT:
251 Table 1, Fig. 3). Exposure to the pesticide, if anything, reduced the heat tolerance (main effect

252 Pesticide) and this tended to be stronger at high DTF (Pest \times DTF: $P = 0.078$). Notably, this Pest \times
253 DTF interaction effect was further shaped by mean temperature and latitude resulting in a
254 significant Pest \times DTF \times MeanT \times Lat interaction (Table 1, Fig. 3). When the high-latitude larvae
255 were kept at their local mean summer temperature of 20°C they only showed a CPF-induced
256 reduction in CTmax at the high 10°C DTF (Tukey: $P = 0.0078$) and not at the other DTFs (both P
257 > 0.45) (Pest \times DTF: $\chi^2_2 = 7.10$, $P = 0.029$). Yet, at 24°C high-latitude larvae consistently suffered
258 a CPF-induced reduction in CTmax (Pest: $\chi^2_1 = 11.33$, $P < 0.001$; Pest \times DTF: $\chi^2_2 = 0.79$, $P =$
259 0.67). Similarly, when the low-latitude larvae were kept at their local mean summer temperature of
260 24°C they only showed a CPF-induced reduction in CTmax at the high 10°C DTF (Tukey: $P =$
261 0.014) and not at the other DTFs (both $P > 0.69$) (Pest \times DTF: $\chi^2_2 = 5.19$, $P = 0.074$). Yet, at 20°C
262 low-latitude larvae tended to consistently suffer a CPF-induced reduction in CTmax (Pest: $\chi^2_1 =$
263 2.74 , $P = 0.098$; Pest \times DTF: $\chi^2_2 = 1.53$, $P = 0.46$).

264 **Discussion**

265 As expected, exposure to the here applied nominal concentration of the pesticide chlorpyrifos (1
266 $\mu\text{g/L}$ CPF) increased mortality, and decreased growth rate and heat tolerance in *I. elegans*
267 damselfly larvae. A CPF-induced reduction in heat tolerance is as predicted by the ‘toxicant-
268 induced climate change sensitivity’ (TICS) concept². A new key finding was that latitude-specific
269 thermal adaptation buffered for the CPF-induced reduction in thermal tolerance in both high- and
270 low-latitude larvae; as a result, the combination of mean temperature and DTF shaped the TICS
271 patterns. Consistent with the ‘climate-induced toxicant sensitivity’ (CITS) concept², warming
272 increased CPF toxicity in terms of an increased mortality (high-latitude larvae) and a decreased
273 growth rate (low-latitude larvae), and the high 10°C DTF increased CPF-induced mortality.

274 *General patterns in heat tolerance and growth rate in the solvent control*

275 Heat tolerance of larvae from both latitudes was higher when acclimated to a higher mean
276 temperature, supporting the general pattern that upper thermal limits increase with higher
277 acclimation temperatures^{57,63,64}. The higher growth rate of low-latitude compared to high-latitude
278 larvae matches previous findings obtained at constant^{9,65} and at fluctuating rearing temperatures²⁹.
279 This reflects their faster life history associated with having multiple generations per year while the
280 high-latitude larvae complete one generation every two years⁶⁶.

281 Warming decreased growth rate only in high-latitude larvae when DTFs were present (5°C
282 and 10°C), while in low-latitude larvae the growth rate was not affected by mean temperature and
283 DTF. This indicates that at 24°C both DTFs exceeded the optimal temperature for growth rate in
284 high-latitude larvae but not in low-latitude larvae, matching latitude-associated thermal adaptation
285 (see also Verheyen and Stoks²⁹) as local mean summer water temperatures are 20°C in the high-
286 latitude populations and 24°C in the low-latitude populations⁴⁵⁻⁴⁷. This DTF-induced growth
287 reduction at 24°C is probably the result of prioritizing allocation of energy to increased metabolic
288 demands for cell maintenance over allocation of energy towards growth^{2,28,67}.

289 *Warming and DTFs increased pesticide toxicity (CITS)*

290 Not only warming but also DTFs increased CPF toxicity in terms of mortality, highlighting that
291 both the predicted increases in mean temperature and in temperature variation under global
292 warming^{34,48} are key drivers of the CITS concept². Our results confirm the general pattern that the
293 toxicity of many pesticides (including CPF) increases at higher mean temperatures^{1,2} (for the study
294 species⁹). Two major mechanisms can explain this increase in pesticide toxicity at higher constant
295 temperatures: an increased pesticide uptake¹¹ and an increased metabolic conversion into more
296 toxic metabolites¹⁰ (for CPF the conversion to CPF oxon⁶⁸). Notably, 4°C warming only increased
297 CPF-induced mortality in high-latitude larvae but not in low-latitude larvae. The latter indicates

298 that local thermal adaptation to higher mean temperatures in low-latitude larvae was able to buffer
299 the negative impact of CPF on mortality.

300 The increased CPF toxicity under the high (10°C) DTF matches the few ecotox studies
301 that integrated DTFs and all showed chemical toxicants becoming more toxic under DTFs in
302 aquatic ectotherms (for pesticides³⁰⁻³², for metals³³). The underlying mechanisms for a higher
303 toxicity under DTFs are poorly understood. The two above discussed major mechanisms
304 underlying the higher CPF toxicity at higher mean temperatures may play a role because during
305 the 10°C DTF cycle larvae were exposed for several hours to high temperatures going up to 25°C
306 at the mean temperature of 20°C and even to 29°C at the mean temperature of 24°C. Moreover,
307 since exposure to DTFs is assumed to be energetically costly for ectotherms (reviewed in Colinet
308 et al.²⁸), less energy will be available for detoxification and damage repair⁶⁹ when exposed to a
309 second stressor like the pesticide CPF. Pesticide toxicity can indeed increase greatly when
310 combined with an energetically costly environmental stressor⁵.

311 Chlorpyrifos also reduced the growth rate but only at 24°C in low-latitude larvae. CPF-
312 induced reductions in growth rate have been observed before in damselfly larvae⁷⁰, including the
313 study species⁹. This can be explained by an energy trade-off between growth rate and defense
314 mechanisms against the pesticide⁶⁹. Since CPF becomes more toxic at higher temperatures¹, at
315 24°C larvae probably allocated more energy away from growth rate towards detoxification (e.g.
316 upregulation of glutathione-S-transferase⁷⁰) and other defense mechanisms (e.g. upregulation of
317 heat shock protein 70⁷⁰). That we did not observe a CPF-induced reductions in growth rate in high-
318 latitude larvae may be explained by the fact that growth rates were already low in the control
319 treatments (because of their slower life history⁶⁶), especially when temperatures got stressful (at
320 24°C with 5°C or 10°C DTF). Moreover, survival selection may have removed the weakest (with

321 the lowest growth rates) high-latitude larvae at 24°C, thereby masking any CPF impact on larval
322 growth rate.

323 *Pesticide exposure decreased the heat tolerance (TICS)*

324 In most thermal treatment combinations, exposure to the pesticide CPF reduced the heat tolerance
325 of both low- and high-latitude larvae. This matches the TICS concept² and is consistent with the
326 general pattern in aquatic ectotherms (in fish¹²⁻¹⁴; in damselflies^{15,16}, including the study
327 species^{17,18}). Heat tolerance is determined by the mismatch between oxygen demand and supply
328 occurring at high extreme temperatures⁷¹. Under toxicant exposure basal metabolism and thereby
329 oxygen consumption are typically higher due to increased energy demands since more energy is
330 needed for detoxification and damage repair mechanisms⁷². In line with this, CPF increased energy
331 costs and negatively affected aerobic performance in the Japanese quail⁷³. Furthermore, in aquatic
332 invertebrates toxicant exposure can impair respiratory functioning which may reduce oxygen
333 uptake (reviewed in Noyes and Lema²). This is likely the case under CPF exposure as it has been
334 shown to damage gills in crabs⁷⁴ and fish⁷⁵. Taken together, the reduced heat tolerance under
335 toxicant exposure can be explained by the mismatch between oxygen demand and supply
336 occurring already at lower temperatures in case animals are exposed to chlorpyrifos⁷².

337 A key finding was that the CPF-induced decrease in thermal tolerance was buffered by
338 local thermal adaptation in both high- and low-latitude larvae. This is the first demonstration that
339 thermal adaptation can shape the TICS patterns by reducing the negative impact of a toxicant on
340 heat tolerance. Specifically, it was the combination of mean temperature and DTF that determined
341 the TICS patterns, thereby adding a new dimension to the importance of DTF in ecotoxicology.
342 Indeed, at the mean summer water temperature at the high latitude (20°C), CPF consistently
343 reduced the heat tolerance in low-latitude larvae across all DTFs, while in high-latitude larvae this
344 reduction only occurred at high (10°C) DTF. Similarly, at the mean summer water temperature at

345 the low latitude (24°C), CPF reduced heat tolerance across all DTFs in high-latitude larvae, while
346 in low-latitude larvae this reduction was only present at high (10°C) DTF. Notably, these
347 combined effects of mean temperature and DTF in shaping the toxicant-induced sensitivity to
348 climate change (TICS), also indicate that the TICS and CITS concepts are not independent (see
349 also Op de Beeck et al.¹⁸).

350 *Implications for risk assessment under global warming*

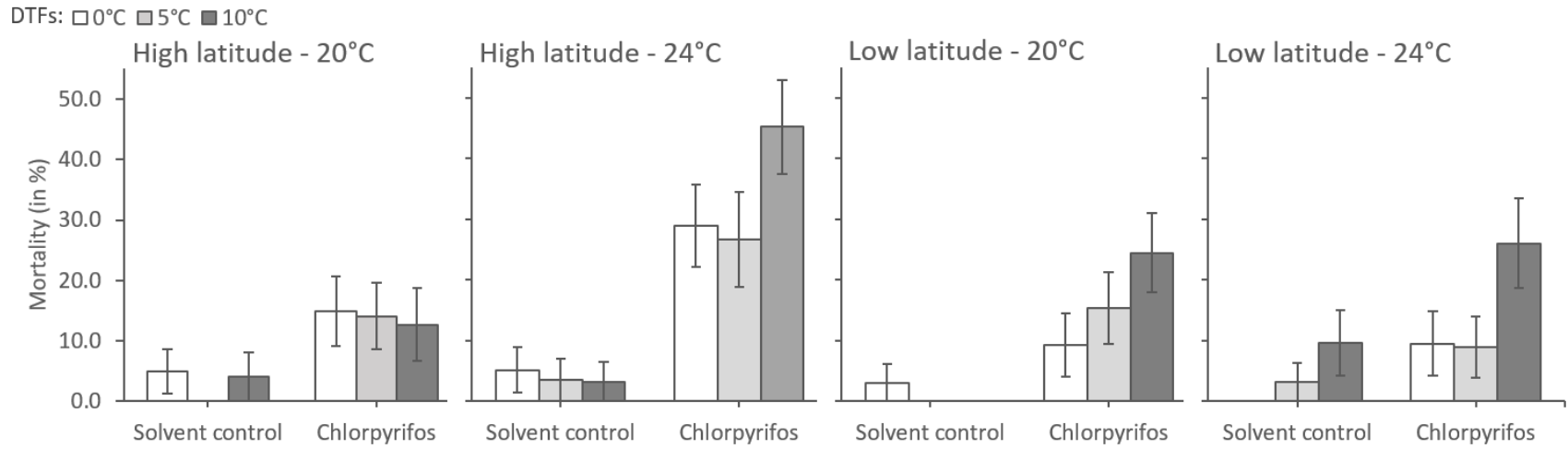
351 Both the TICS and CITS concepts are central to our understanding of the effects of toxicants in a
352 warming world^{2,4}. By studying in an integrated way the single and combined effects of 4°C
353 warming and an 5°C increase in DTF on chlorpyrifos toxicity, and vice versa the effect of
354 exposure to CPF on the sensitivity to warming, at two strategically chosen latitudes, we added
355 important novel insights to both concepts. We provided proof-of-principle that besides an increase
356 in mean temperature also higher DTFs under global warming are not only a key driver of CITS (by
357 increasing mortality and decreasing larval growth rate), but more importantly also of the
358 interdependence of both concepts. Notably, other conclusions would have been reached about both
359 the strengths of both concepts when ignoring DTF. This connection between the TICS and CITS
360 concepts differed between latitudes, reflecting a crucial role of latitude-associated thermal
361 adaptation. This highlights an overlooked geographic component in how DTF may shape toxicant
362 sensitivity and the connectedness of the TICS and CITS concepts.

363 Our results imply that taking DTF into account may be crucial to arrive at realistic
364 predictions of the effect of global warming on toxicant sensitivity. Indeed, while our results
365 suggest that gradual thermal evolution in high-latitude larvae may buffer the effects of CPF on
366 heat tolerance under warming, this is no longer true when also taking the increase in DTF into
367 account. This prediction is based on a space-for-time substitution^{20,25} taking into account that the
368 current thermal regime at the low latitude (mean of 24°C and maximum DTF of 10°C) matches the

369 future thermal regime at the high latitude under global warming by 2100, as predicted by the IPCC
370 (2013)⁴⁸ RCP8.5 scenario. When assuming no thermal evolution, high-latitude larvae would suffer
371 a CPF-induced decrease in heat tolerance under 4°C warming (24°C) at all DTFs. However, under
372 the more realistic scenario where high-latitude larvae could gradually evolve to have the same
373 ability as low-latitude larvae to deal with a higher mean temperature (24°C), high-latitude larvae
374 would no longer suffer a CPF-induced reduction in heat tolerance under 4°C warming unless we
375 also take into account the expected increase to 10°C DTF.

376 To conclude, we demonstrated that the daily temperature fluctuations which most
377 organisms encounter in nature are a key environmental factor shaping contaminant toxicity,
378 making them highly relevant for current and future risk assessment of toxicants. The novel finding
379 of latitude-associated thermal adaptation in how DTFs shape toxicity, adds an important but
380 overlooked dimension to the calls to take spatial variation in local conditions into account during
381 risk assessment of toxicants^{21,76}. Moreover, we provided the first evidence that DTFs, that are
382 expected to increase under global warming²⁷, are highly relevant to consider at the interface of
383 evolutionary ecotoxicology and global change biology. Indeed, our results indicate that predictions
384 of the future impact of CPF under global warming may be critically dependent on the inclusion of
385 DTF. Taken together, our results highlight the importance of integrating both daily temperature
386 fluctuations and a geographic component to improve both current and future risk assessment of
387 chemical toxicants under global warming^{2,7}.

388 **Figure 1**



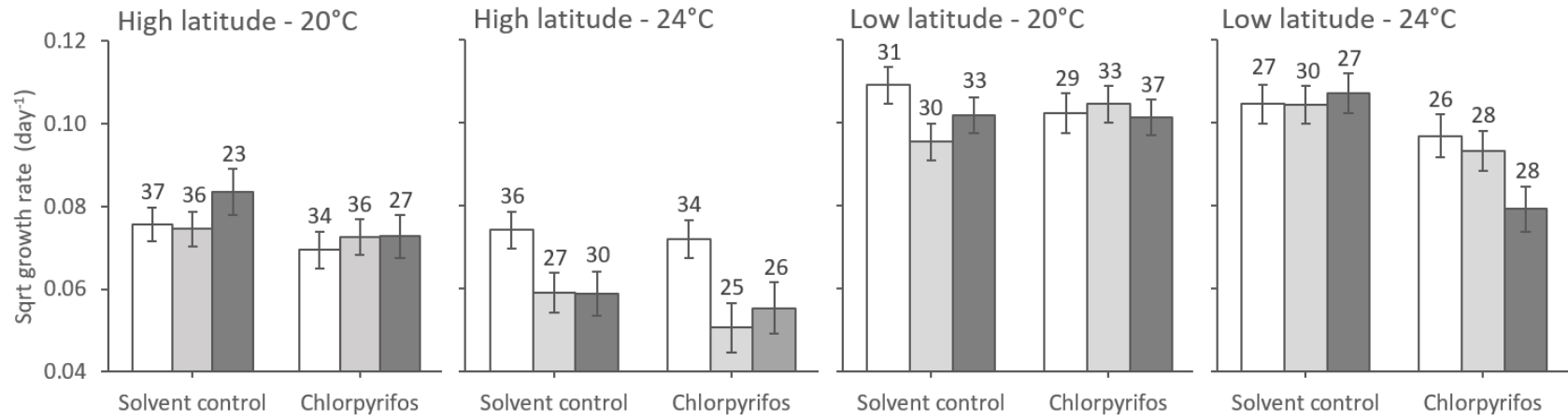
389

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

392

393 **Figure 2**

DTFs: □ 0°C □ 5°C ■ 10°C



394

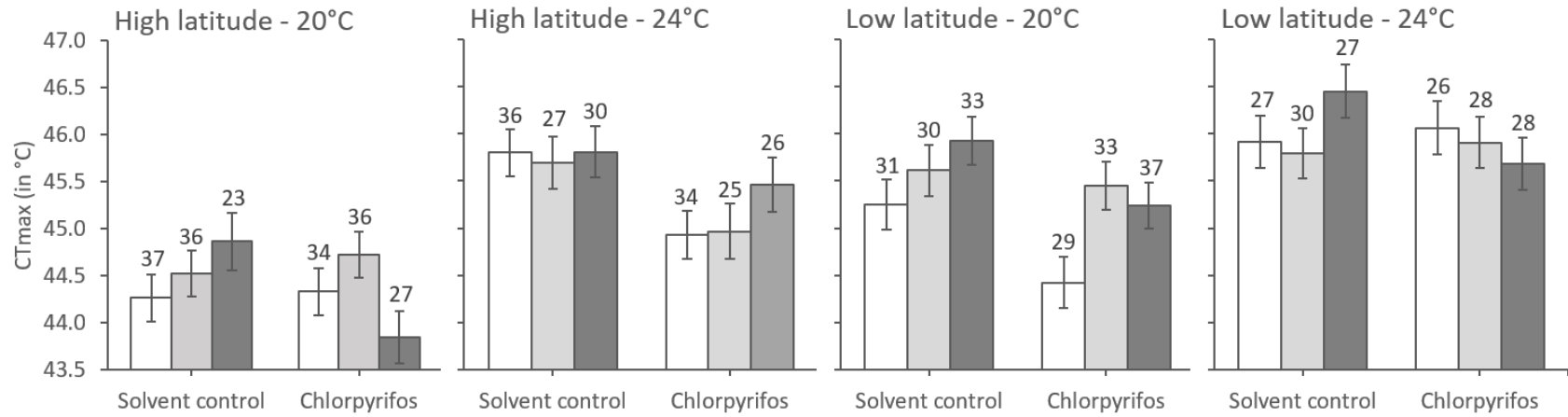
395 **Figure 2.** Mean (± 1 SE) growth rate of high-latitude and low-latitude *Ischnura elegans* damselfly larvae as a function of pesticide
396 exposure, daily temperature fluctuation (DTF) and mean temperature. Numbers above bars represent sample sizes.

397

398

399 **Figure 3**

DTFs: □ 0°C ■ 5°C ■ 10°C



400

401 **Figure 3.** Mean (\pm 1 SE) heat tolerance (measured as CTmax) of high-latitude and low-latitude *Ischnura elegans* damselfly larvae as a
402 function of pesticide exposure, daily temperature fluctuation (DTF) and mean temperature. Numbers above bars represent sample sizes.

Table 1. Results of linear mixed models testing for the effects of pesticide exposure (Pest), daily temperature fluctuation (DTF), mean temperature (MeanT) and latitude (Lat) on larval growth rate and heat tolerance (CTmax) in *Ischnura elegans* damselfly larvae. Body mass was added as covariate in the heat tolerance model. Bold *P*-values are significant (< 0.05).

Effect	Growth rate			Heat tolerance		
	χ^2	df	<i>P</i>	χ^2	df	<i>P</i>
Pest	10.75	1	0.0010	11.79	1	<0.001
DTF	8.43	2	0.015	5.26	2	0.072
Mean T	20.71	1	<0.001	61.80	1	<0.001
Lat	258.48	1	<0.001	43.01	1	<0.001
DTF × Mean T	6.82	2	0.033	4.91	2	0.086
Pest × Mean T	3.45	1	0.063	0.41	1	0.52
Pest × DTF	2.39	2	0.30	5.10	2	0.078
Pest × Lat	0.26	1	0.61	0.37	1	0.54
Mean T × Lat	4.34	1	0.037	3.33	1	0.068
DTF × Lat	1.51	2	0.47	0.72	2	0.70
Pest × DTF × Mean T	2.81	2	0.24	0.51	2	0.77
Pest × Mean T × Lat	5.08	1	0.024	2.95	1	0.085
Pest × DTF × Lat	1.34	2	0.51	0.54	2	0.76
DTF × Mean T × Lat	6.75	2	0.034	4.46	2	0.11
Pest × DTF × Mean T × Lat	2.39	2	0.30	7.19	2	0.028
Body mass	/	/	/	7.48	1	0.0063

404 **Supporting Information**

405 Appendix S1: Realized thermal regimes

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409 **Authors' Contributions**

410 JV and RS conceived and designed the experiments. JV performed the experiments and analyzed
411 the data. All authors wrote the manuscript and gave final approval for publication.

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420 manuscript.

421 **Abbreviations**

422 BCC_CSL 1.1, Beijing Climate Center Climate System Model 1.1; CITS, Climate-Induced
423 Toxicant Sensitivity; CPF, chlorpyrifos; CTmax, critical thermal maximum; DTF, daily
424 temperature fluctuation; IPCC, Intergovernmental Panel on Climate Change; Lat, latitude;
425 MeanT, mean temperature; NOEC, No Effect Concentration; Pest, pesticide; RCP 8.5,
426 Representative Concentration Pathway 8.5; TICS, Toxicant-Induced Climate Change sensitivity.

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