



Article

Investigation of the Combined Effects of Rising Temperature and Pesticide Contamination on the Swimming Behaviour of Alpine Chironomids

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Abstract: Some pollutants can be transported through the atmosphere and travel medium–long distances to be deposited in glaciers at high altitude and latitude. The increase in the rate of glacier melting due to global warming can release these pollutants in alpine streams. This study investigated the combined effects of rising temperatures and chlorpyrifos (CPF) contamination on the swimming behaviour of alpine chironomids collected in a shrinking alpine glacier. We assessed the individual and interaction effects of rising temperatures (2–11 °C) and CPF concentrations (0–110 ng L⁻¹) on the swimming behaviour of *Diamesa zernyi* (Chironomidae) larvae. Distance (mm) and speed (mm s⁻¹) were recorded using a video-tracking system after 24–72 h of treatment. The two stressors caused different effects on distance and speed, with increasing temperature generally causing hyperactivity and CPF from hyperactivity to reduced mobility. Two interactions were detected between stressors when combined: (i) CPF superimposed the effect of temperature on both behavioural endpoints i.e., with 110 ng L⁻¹ of CPF, at 11 °C, larvae moved less; (ii) warming (11 °C) magnified the negative effect of CPF: the smallest distance and slowest speed were recorded at the highest values of the two stressors after 72 h. Our results suggest that water contamination by CPF, even at sub-lethal concentrations, might increase the sensitivity of chironomids to warming, and vice versa, raising concerns about freshwater biodiversity conservation under climate change.

Keywords: *Diamesa zernyi*; chlorpyrifos; distance; speed; video tracking; glacier-fed streams; Italian Alps; climate change



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1. Introduction

The quantity and quality of freshwater is threatened by climate change and pollution [1]. Climate change is leading to the warming of rivers and lakes and shrinking of the glaciers, which in turn is causing a deterioration of melting water quality. This is because glaciers are a temporary sink of inorganic and synthetic organic substances transported from the human settlements in lowlands and mountain valleys to higher altitudes. These pollutants accumulate in the ice and snow due to cold condensation, and are released in melting waters entering the streams [2–7]. Among these are pesticides (such as chlorpyrifos, captan, terbuthylazine), synthetic fragrances (such as galaxolide and tonalide), persistent organic pollutants, and polycyclic aromatic hydrocarbons [7]. Understanding how alpine freshwater species may react and adapt to increasing temperatures and pollutants is a major challenge for hydrobiologists and ecologists that are looking to predict future aquatic biodiversity trends in mountain regions [8,9]. The extent to which highly specialised species living in extreme habitats such as glacier-fed streams are threatened by extinction due to climate change is widely debated [10]. These habitats host simplified animal communities dominated by cold stenothermal chironomids of the genus *Diamesa* (Diptera Chironomidae), highly specialised to survive freezing, low food availability, and high discharge and

turbidity [11,12]. Studying the adaptive potential of these species to water heating and pollution of streams fed by shrinking glaciers is a priority for understanding the real risk of extinction of alpine invertebrate species [13–15].

As there is evidence that warming and pesticides can interact synergistically [16,17], we established a laboratory experiment to assess the combined effects of rising temperature and chlorpyrifos (CPF) contamination on the larval behaviour of *Diamesa zernyi* (Edwards, 1933) as a representative of cryophilous fauna, that are almost restricted to glacier-fed streams [18]. *Diamesa* spp. is not a traditional ecotoxicological model species [19], and no toxicity tests from the OECD Guidelines are available for species belonging to this genus. *D. zernyi* is a cold stenothermal species, whose larvae have a thermal optimum of 3.7 °C [20] and are freeze-tolerant (supercooling point = −6.3 °C; lower lethal temperature = −16.2 °C) [21]. Recent studies have highlighted that, larvae of this, and other *Diamesa* species, tolerate 96 h exposure to pharmaceuticals (furosemide, ibuprofen, and trimethoprim), surviving concentrations millions of times higher than those in the environment, but are susceptible to pesticides (CPF, metolachlor, and captan), with significant lethal effects after 24–48 h of exposure [15]. Some authors [22,23] have highlighted that among these pesticides, CPF, at an environmental temperature of 2 °C, affects the swimming behaviour of *D. zernyi* larvae at LC10,48 h (1.1 µg L^{−1}) and alters their metabolism [24]. However, no information is available on the combined effects of rising temperature and CPF on the behaviour of this species.

We selected swimming behaviour as the endpoint as locomotor performance is an ecologically relevant trait. Recent studies have demonstrated that aquatic organisms can survive when exposed to sub-lethal concentrations of contaminants, but can undergo behavioural changes (e.g., escaping to avoid contamination, changing eating habits, or the mode of locomotion) [25,26]. Behavioural disturbances of key species might also affect ecosystem processes [27], for example, by altering prey–predator interactions or competition for food and space [25,26]. Changes to behaviour involve an integrated whole-organism response that often incorporates early stress signals produced at the sub-organismal level, including changes to biochemical, cellular, and/or physiological levels [23,28]. Sub-lethal endpoints are sensitive to low pollutant concentrations and act as early warning tracers of environmental changes [28–30].

In our study, we experimentally tested the hypothesis that the two stressors interact with each other, affecting the behaviour of *D. zernyi* larvae in a different way than their individual effects. *D. zernyi* larvae were exposed for 72 h to different combinations of environmentally relevant concentrations of CPF, selected according to [7,15], and temperatures higher than the environmental temperatures, selected according to [31]. We expected a major effect at the highest temperature and pesticide concentration in a synergistic manner.

2. Materials and Methods

2.1. Test Species

All laboratory experiments were conducted on the fourth-instar larvae of *D. zernyi* collected from the Rio Presena stream (46°13.596' N; 010°34.929' E) at 2685 m above sea level. The stream is fed by the Presena glacier (Noce River catchment, Trentino Province, NE Italy).

The larvae were collected with a 30 cm × 30 cm pond net (mesh size 250 µm) (Scubla SNC, Italy) within 25 m from the glacier snout, on five occasions in the late summer of 2017 (7, 11, 12, 14, and 18 September 2017). Larvae were gently sorted in the field with tweezers to minimise impact or damage to the larvae, transferred to 100 mL plastic bottles filled with stream water, and transported to the laboratory in a cooling bag. Species were identified within 24 h of sampling, and all larvae were checked under a stereomicroscope (MZ 7.5; Leica Microsystems, Wetzlar, Germany; 50×) following [32]. All collected larvae were maintained in 1 L glass aquaria (approximately 500 larvae/aquarium) with stream water in a thermostatic chamber (ISCO, model FTD250-plus; Teledyne Isco Inc., Lincoln, NE, USA) maintained at 2 °C with aeration to maintain dissolved oxygen higher than

80% saturation, under a specific photoperiod (8 L:16 D). The incubation temperature (2 °C) was the water temperature measured in the stream using a Hydrolab Quanta (Hydrolab Corporation, Austin, TX, USA) multi-parametric probe at the first sampling date (7 September 2017). To acclimate the larvae to the exposure conditions, randomly selected larvae were transferred 24 h prior to each experiment to a 500 mL beaker (approximately 40 larvae/beaker) containing 200 mL of soft reconstituted water (RW) with the following composition: 4.36 mg L⁻¹ NaHCO₃, 2.73 mg L⁻¹ CaSO₄ 2H₂O, 2.73 mg L⁻¹ MgSO₄, and 0.19 mg L⁻¹ KCl (pH = 7.7) [12]. During acclimation and exposure, the larvae were maintained at 2.0 ± 0.1 °C without food, in aerated water.

2.2. Test Solutions

The following three CPF concentrations were tested: 1.1, 11, and 110 ng L⁻¹. They correspond to values three, two, and one order of magnitude lower than the LC10,48 h of 1100 ng L⁻¹, estimated at 2 °C by [15]. Additionally, the intermediate value (11 ng L⁻¹) corresponds to the mean CPF concentration in the Presena stream in summer (9.43 ± 13.0 ng L⁻¹) [7], comparable with the level of CPF contamination in other Alpine ice melt waters [6]. Each of the CPF (purity > 95%, Sigma Aldrich Laboratories, Milan, Italy) concentrations were prepared in RW and ethanol (analytical purity). Ethanol was added to a final concentration of <0.1 mg L⁻¹ to favour CPF solubilisation in water. The percentages of co-solvent used in the final test samples was 1.1 × 10⁻⁴%, 1.1 × 10⁻⁵%, and 1.1 × 10⁻⁶% in volume (*v/v*%). These percentages were well below the guidelines suggested by [33] (≤0.1 mg L⁻¹) and were tested in order to verify the existence of a co-solvent effect on organism behaviour. Data obtained for the control groups of larvae exposed to the three concentrations of co-solvent ethanol confirmed that there were no effects of ethanol on larval survival and behaviour at 2 °C without CPF (Tables S1 and S2), validating our experimental design. Given the absence of ethanol effects on survival and behavioural endpoints, in all experiments, the control consisted of RW plus the highest ethanol concentration. The test solutions were renewed every 24 h. The stability of CPF in water has been tested previously [23] at 2 °C and 72 h from the addition of 524 ng L⁻¹ of CPF. After 72 h, the CPF concentration was 520 ng L⁻¹. Under such experimental conditions, the nominal concentration of CPF did not change significantly (with a relative standard deviation lower than 20% among replicates) during the experiments, in accordance with [23].

2.3. Experimental Procedures

The control (RW + ET) and test larvae were placed in thermostatic chambers and exposed for 72 h to three temperatures, 2.0 ± 0.1, 8.0 ± 0.1, and 11.0 ± 0.1 °C. The lowest value (2 °C) corresponded to the mean environmental temperature during the sampling period. The highest value (11 °C) was selected as the mean temperature of glacial streams fed by shrinking glaciers in the same area [12], which is still non-lethal in the short-term (100% survival was observed after 3 h at 15 °C by [31]). The temperature of 11 °C is higher than the thermal optimum for this species (3.7 °C; [20]). Finally, 8.0 °C was selected as the intermediate temperature between the other two values. Larval survival was checked every 24 h during the experiment. For each temperature and CPF concentration, a battery of three replicates, each containing three larvae, were taken. We treated nine organisms for CTRL_ET1, CTRL_ET2, CTRL_ET3, CPF_1.1, CPF_11, and CPF_110 ng L⁻¹ at each temperature of exposure (Figure 1). Thus, we treated 54 organisms for all treatments at each exposure temperature, with a total of 162 organisms (54 organisms × 3 temperatures). Every 24 h, three repeated measurements were recorded using a video-based tracking system in groups of three larvae at a time (up to nine larvae) in a beaker filled with the same exposure medium. After recording, the larvae were placed back in the glass vessels used for the exposure. Finally, 27 measurements were taken for each treatment (i.e., each combination of chemical/control plus temperature of exposure). All treatments, each with the respective control, were conducted simultaneously in a single experiment for 72 h. For each temperature, a battery of nine replicates with CPF (3 × each concentration) and

three control replicates, with three larvae each, were used. Every 24 h, a sub-group of one to three larvae (only live larvae were considered) was taken from each replicate and tested. Three repeated measurements were recorded for each larva using a video-based tracking system.

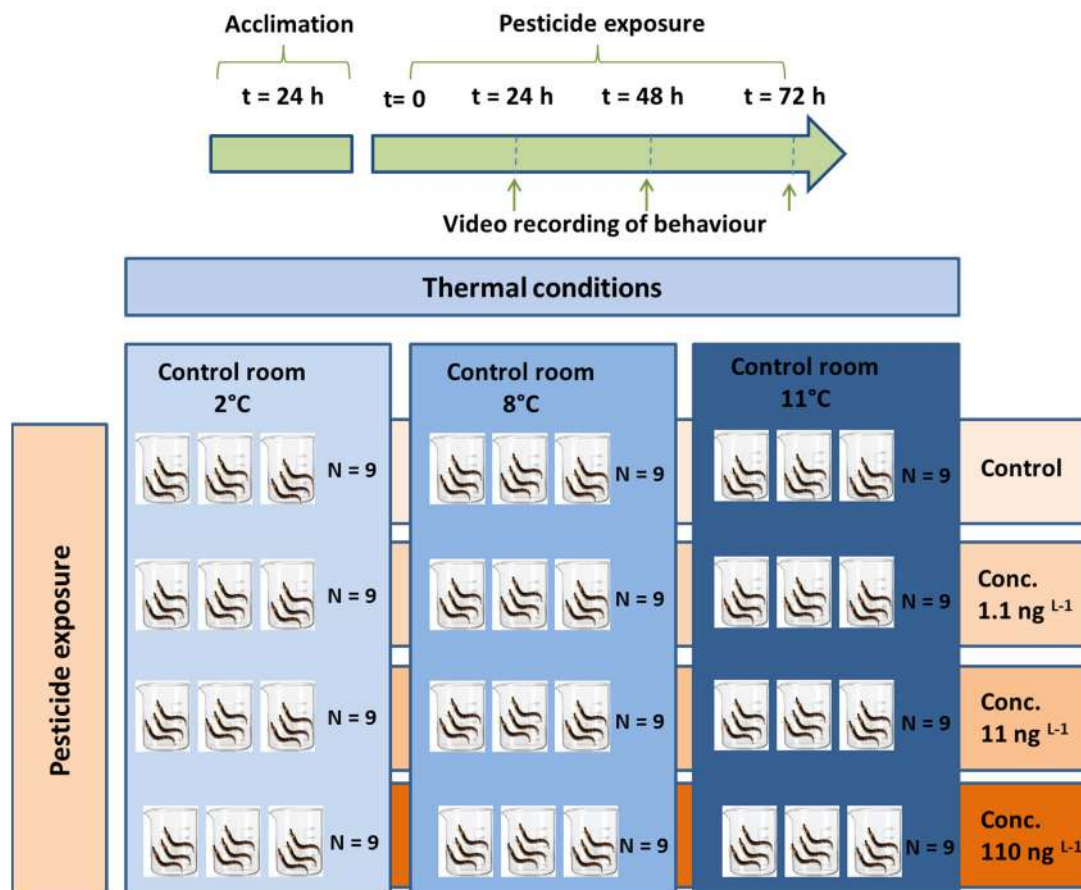


Figure 1. The experimental design.

Video footage was taken after 24, 48, and 72 h of exposure under different combinations of temperature and pesticide concentration to monitor the time effect on behaviour. A prolonged exposure time of up to 72 h in comparison to standardised test protocols (up to 48 h) [22] was considered as it is known that in cold-adapted organisms, the expression of toxicity is delayed owing to slow CPF uptake kinetics and a slow metabolic rate [34]. Furthermore, data on behaviour were recorded every 24 h for 3 days as contamination is sometimes characterised by short-term peak concentrations, such as when pesticides are applied to crops [35,36]. CPF has a high value of K_{ow} (octanol/water partition coefficient, $\log K_{ow} = 4.7$, according to the PPDB: Pesticide Properties DataBase), which may be responsible for its delayed passage through the cell membrane [37].

All videos were recorded for 180 s at high resolution (1920×1080 pixels) using a digital high-definition camera (Raspberry Pi 3 with Camera Module v2) positioned 10 cm from the bottom of each beaker. During this time, the water temperature varied between $+0.1$ to $+0.3$ °C, as recorded using a portable thermometer (Koch 13211; ± 1 °C). Behavioural effects were assessed.

Video processing and analysis were conducted using the FFmpeg (900 frames; 30 frames per second) (<http://www.ffmpeg.org> (accessed on 21 September 2017)) and ImageJ software (<http://imagej.nih.gov/ij/> (accessed on 21 September 2017)), respectively, following [19,22]. The software was optimized/calibrated according to [19] to monitor the distance and average speed of the (the behavioural endpoints) of the *Diamesa* larvae. Data

on distance and speed were converted from pixels to mm and from pixels s⁻¹ to mm s⁻¹, respectively, according to the procedure described in [22]. An example is given in Video S1 (“*Diamesa zernyi* swimming”).

2.4. Statistical Analysis

The normality and homogeneity of variances were tested using the Kolmogorov-Smirnov and Leven’s tests, respectively. Distance and speed were normally distributed (K-S $p < 0.05$), and survival proportion data were normalised (arcsine-square-root-transformation) following [38]. Statistically significant effects of temperature and CPF on larval survival and behaviour after the three different exposure periods were investigated using two-way analysis of variance (ANOVA). The results of each treatment group were compared with those of the control group and treatment level differences using Dunnett’s multiple comparisons test. The data shown in Figures A2–A4 were normalised by setting the untreated control group (untreated larvae) as 1.

Statistical analyses were conducted using GraphPad Prism 6 (version 6.01; 2012), SPSS 24 (IBM), and STATISTICA (version 12.0; 2013 © StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Survival under Thermal Stress and CPF Exposure

Without CPF, larvae were found to be alive after 24 h, independent of temperature (Table 1 and Table S3), and within the acceptable range $\leq 15\%$ set by the OECD guideline n. 235, that refers to the *Chironomus* spp. [39], throughout the test duration at the optimal thermal regime of 2 °C. Increased mortality was recorded with increasing the time of exposure and at higher temperatures, for example, after 72 h at 8 and 11 °C.

Table 1. Mean (\pm standard error) percent survival of the *Diamesa zernyi* larvae exposed to increasing temperatures (2, 8, and 11 °C) and chlorpyrifos (CPF) concentrations (0, 1.1, 11, and 110 ng L⁻¹), for 24, 48, and 72 h.

	CTRL 2 °C	CTRL 8 °C	CTRL 11 °C
24 h	100 \pm 0.0	100 \pm 0.0	100 \pm 0.0
48 h	100 \pm 0.0	89 \pm 11	67 \pm 17
72 h	89 \pm 9	78 \pm 15	56 \pm 17
CPF (2 °C)			
	1.1	11	110
24 h	100 \pm 0.0	100 \pm 0.0	100 \pm 0.0
48 h	100 \pm 0.0	89 \pm 11	89 \pm 11
72 h	100 \pm 0.0	44 \pm 18	56 \pm 18
CPF (8 °C)			
	1.1	11	110
24 h	100 \pm 0.0	100 \pm 0.0	100 \pm 0.0
48 h	100 \pm 0.0	89 \pm 11	100 \pm 0.0
72 h	89 \pm 11	78.0 \pm 15	100 \pm 0.0
CPF (11 °C)			
	1.1	11	110
24 h	100 \pm 0.0	100 \pm 0.0	100 \pm 0.0
48 h	100 \pm 0.0	78.0 \pm 15	67 \pm 17
72 h	100 \pm 0.0	67 \pm 17	44 \pm 18

The addition of CPF at 2 °C caused significant mortality ($>15\%$) at concentrations of 11 (survival = 44 \pm 18%) and 110 (survival = 56 \pm 18%) ng L⁻¹ after 72 h. The highest mortality (44 \pm 18%) was observed after 72 h at 11 °C and 110 ng L⁻¹.

Survival values recorded after 72 h at the intermediate and highest CPF concentrations were outside the acceptable limit of mortality set by the OECD guidelines [33]. Despite

this, behaviour of the surviving larvae was analysed under these experimental conditions to add information on the stress response at the individual level at concentrations and temperatures to identify the upper surviving limits for this species. As the surviving larvae were not found moribund, we considered valid results on behaviour even under such experimental conditions.

3.2. Behaviour under Thermal Stress and CPF Exposure

Without CPF, distance and speed increased with respect to the control (2 °C) after only 24 h of exposure (Figure A1). For distance, this increase was significant between 2 and 8 °C at 24 h, and between 2 and 11 °C and 8 and 11 °C at 72 h of exposure (Figures A1A and 2). The increase in speed was significant between 2 and 11 °C at 24 h, between 8 and 11 °C at 48 h, and between 2 and 11 °C and 8 and 11 °C at 72 h of exposure (Figure A1B). Overall, higher values of distance and speed (independent of temperature) were observed at 24 h (both) and 48 h (only speed).

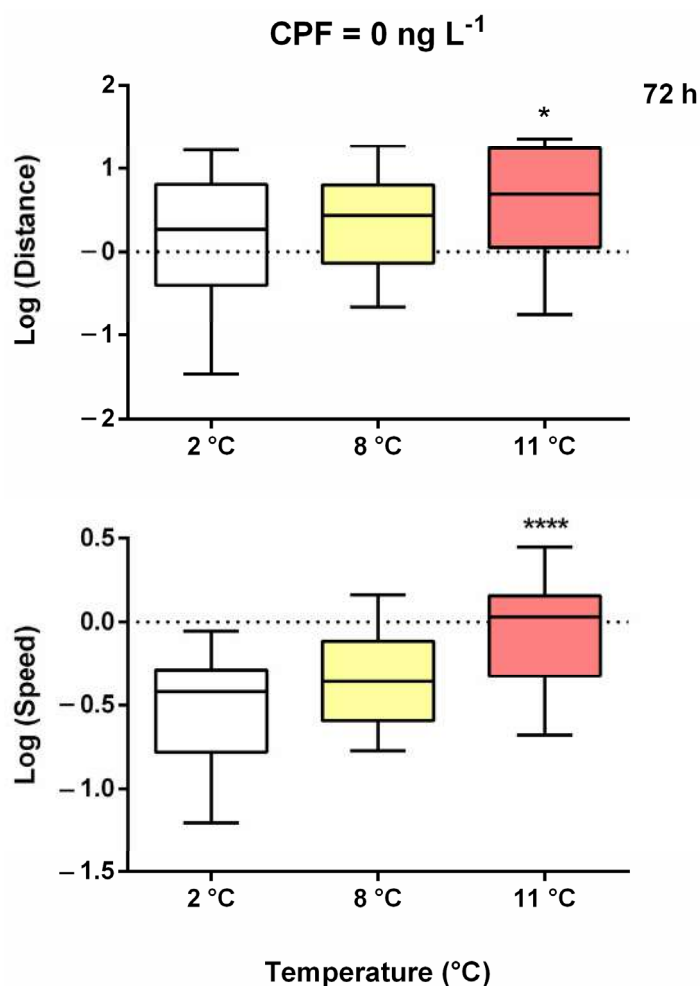


Figure 2. Boxplots of the log-transformed median distance (**up**) and speed (**down**) under increasing thermal stress after 72 h of treatment. Asterisks = significant differences from the control group * $p < 0.05$; **** $p < 0.0001$.

The addition of CPF at 2 °C caused a significant negative effect only on speed after 72 h of exposure at the highest concentration (110 ng L⁻¹) (Figures A2B and 3).

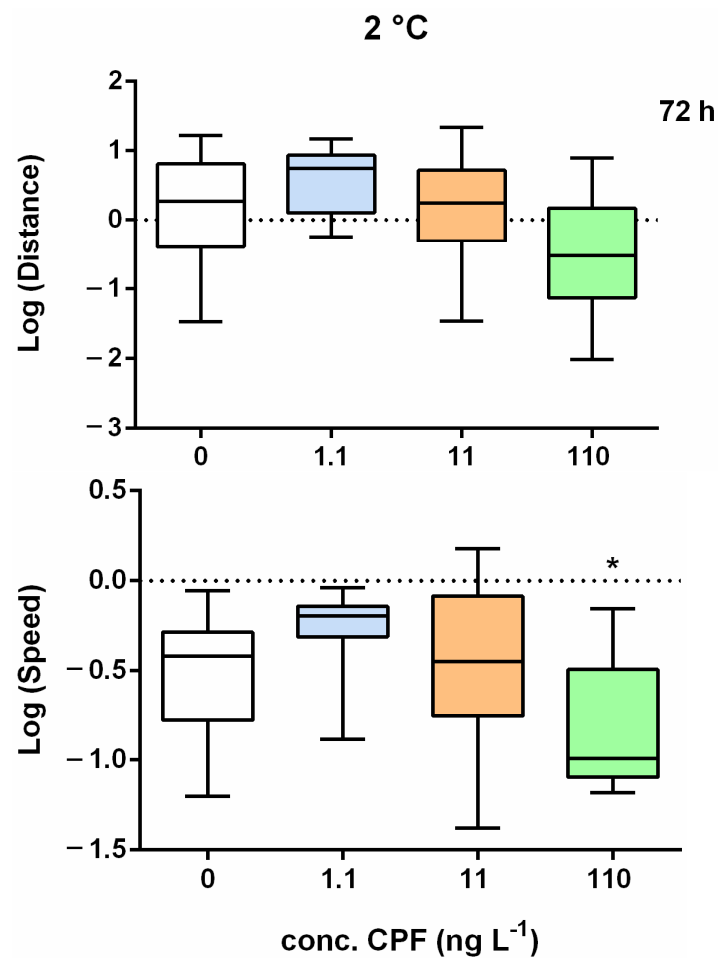


Figure 3. Boxplots of the log-transformed median distance (**up**) and speed (**down**) under an increasing chemical stress after 72 h of treatment. Asterisks = significant differences from the control group * $p < 0.05$.

Under multiple stresses (Figures A3 and A4) after 24 h, significant effects were observed only on speed and were higher after 24 h at 8 °C with 1.1 ng L⁻¹. At 11 °C, speed was lower than that at 8 °C, with the lowest CPF concentration (Figure A4A). After 48 and 72 h of exposure, significant positive and negative effects were detected, respectively, for distance and speed. After 48 h, the distance generally increased at 11 °C with 11 ng L⁻¹ and at 8 °C with 110 ng L⁻¹ (Figure A3B). A similar trend was observed for speed, which was higher than in the control with 11 ng L⁻¹ at 8 and 11 °C and with 110 ng L⁻¹ at 8 °C (Figure A4B). After 72 h (Figure 4), the most evident change was the reduction in distance (Figure A3C) and speed (Figure A4C) at 11 °C with 110 ng L⁻¹ of CPF.

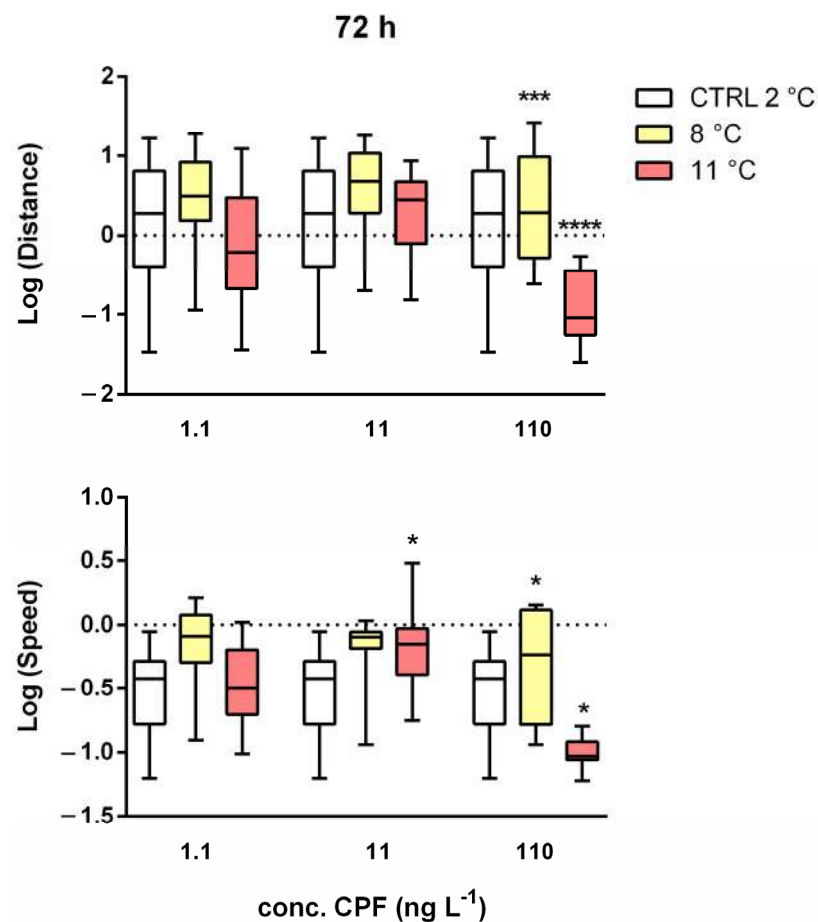


Figure 4. Boxplots of the log-transformed median distance (**up**) speed (**down**) variable against concentration and temperatures after 72 h of treatment. Asterisks = significant differences from the control group * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

4. Discussion

Hydrological alterations, habitat degradation and loss, pollution, overexploitation, invasive species, and climate change have been detected as major threats to freshwater ecosystems [40]. Each category includes multiple stressors to biodiversity that interact, often with effects greater than (synergism), less than (antagonism), or equal to the sum of their individual effects [41,42]. Scientists have sought to identify the most significant causes of stress, understand and predict the responses of species and communities to individual and multiple-stressor configurations [43,44], support administrations in developing sustainable management actions, and finding nature-based solutions according to the 2030 Agenda for Sustainable Development (<https://www.un.org/sustainabledevelopment/development-agenda/> (accessed on 5 September 2021)). The results of our study are relevant in this context, highlighting, for the first time, the interaction between the two stressors (global warming, and chemical pollution) on biodiversity in remote alpine areas. To predict the impact of pesticides under global warming, we need to understand how species respond to the interaction between the two stressors and whether their sensitivity to increasing temperature can be influenced by chemical stressors and vice versa [45]. This is the first time that temperature and chemical stress interactions have been experimentally tested using wild insect species from glacier-fed streams.

Temperature and CPF separately also affected survival. In particular, at 11 °C, CPF concentrations of 11 and 110 ng L⁻¹ had detrimental effects on larval survival. Unlike what was observed for shorter periods of exposure time [15,31], at 11 °C, the concentrations of CPF at 1/100 to 1/10 of the LC₁₀,48 h were not sub-lethal even after prolonged exposure (72 h). Between 24 and 48 h of exposure, survival remained $\geq 89\%$ between CPF concen-

trations of 0 and 110 ng L⁻¹, which is within the acceptable criteria for chironomids [39], confirming these as sub-lethal values within 48 h of exposure. This evidence supports [34] the delayed toxicity of CPF, particularly for organisms from cold freshwater. This is relevant for *Diamesa* species under the continuous release of pesticides from the glacier during the melting season [7,36], corresponding to their biological window [20].

Under the two stressors, at the highest temperature (11 °C) and concentration (110 ng L⁻¹), survival after 72 h was comparable to that under the two stressors separately. These results suggest that chemical and thermal stresses do not have an additive effect on survival, as the mortality registered under both stress conditions at 48 or 72 h were only slightly higher than those observed under a single stress.

Regarding behaviour, temperature had a positive effect on both endpoints, obtaining almost double the values of the control at 11 °C than at 2 °C independently of the time of exposure. In ectotherms, basal metabolism (i.e., oxygen consumption) increases at temperatures higher than the range in which the species normally lives [46], which could justify the hyperactivity of the larvae. Considering that significant mortality occurred after 72 h at 11 °C, this might be considered as the temperature near the upper limits of thermal tolerance. Near this limit, oxygen is typically limiting, affecting all higher biological functions (i.e., muscular activity, growth, and reproduction), thereby affecting survival and, at long-term, the fate of species [47].

The addition of CPF to larvae kept at 2 °C did not show any effects, with the sole exception of a significant decrease in speed after 72 h at 110 ng L⁻¹. This suggests that at 2 °C, CPF does not affect larval behaviour in the short term.

Under multiple stresses, hyperactivity and immobility were observed at different times of exposure, highlighting the interactions between the two stressors in a different way in terms of distance and speed. Speed was the first to show a change in the endpoint. In the short term (24 h), we observed only an increase in speed, at 8 °C, in the presence of 1.1 ng L⁻¹ CPF. This could be caused by tremors in the larvae, which suggested an initial state suffering. After 48 h, we observed an increase in both distance and speed with concentrations ≥ 11 ng L⁻¹, particularly at 8 °C (at 11 °C only with an intermediate concentration of 11 ng L⁻¹). Hyperactivity is de facto an escape reaction (avoidance). It can be an adaptive response to avoid stress conditions [48] associated with low-dose stimulation and high-dose inhibition [49]. Larvae might move to find refuge in the hyporheic zone. In fact, a consistent number of studies have suggested that, in all streams, the hyporheic zone is used by benthic invertebrates as a refuge during floods, droughts, or other stressful conditions [50,51]. The strongest effect of the combination of high temperature (11 °C) and high CPF concentration (110 ng L⁻¹) was evident after three days of treatment, with a significant decrease in both distance and speed. The immobilisation of the larvae was probably due to the neurotoxicity of CPF, which inhibits the enzyme acetylcholinesterase, interfering with neural transmission [52–54].

Summarizing, these results verified our hypothesis, emphasising two interactions between the two types of stress. First, CPF superimposed the effect of temperature on both behaviour endpoints, i.e., without CPF, larvae moved faster at higher temperatures but with the pesticide, larvae moved less even at very low concentrations and at 11 °C. Second, warming (11 °C) magnified the negative effect of the toxicant [17,55]. The lowest values for both endpoints were recorded under maximum values of both the stressors (Video S1 “*Diamesa zernyi* swimming”). In such conditions, the basal metabolism of the larvae was probably reduced and, consequently, no attempt to escape was observed. We might argue that organisms devote energy in developing a detoxification response in a physiological state of stress [15,19,22–24,56]. CPF and other organophosphate insecticides are known to exhibit increased toxicity in invertebrates at elevated temperatures [57,58], with effects on survival and behaviour (e.g., altering the escaping speed of the prey of damselflies even at concentrations that current legislation considers environmentally protective [59]).

Additionally, warming influences CPF uptake in aquatic insects [60], increasing its permeability across cell membranes, promoting a higher uptake, and activating a detox-

ification response [34]. Our study emphasised that warming changes the impact of CPF (i.e., magnifying the negative effect) on behaviour and CPF changes the species sensitivity to warming (i.e., inverting the effect on behaviour), indicating that global warming may magnify the impact of pesticides [16,17]. A temperature higher than the thermal ecological optimum, without the insecticide, altered the behaviour of *D. zernyi* larvae, increasing the movement likely related to increasing basal metabolism [14]. When high, but not lethal, temperature was combined with high but sub-lethal concentrations of CPF, higher temperatures seem to strengthen the negative effect of the contaminants on behaviour, making it more bioavailable [61].

Some authors [62] have reported that increased energy demand for maintenance of basal metabolism that is not met by increased generation of energy is central to the stress effects caused by temperature and pollutants. Therefore, there is a likely physiological mechanism underlying how pollutants shape thermal tolerance. Evidence of pesticide-induced climate change sensitivity and warming-induced pesticide change sensitivity has been observed in eurythermal dragonflies [17], but with warming reducing the negative effect of pesticides on sensitivity (these experiments were conducted at 20–24 °C, at which the degradation rate of pesticide is higher than that at our experimental temperature range).

5. Conclusions

In 2020, the European Commission formally adopted regulations proposing not to renew the approvals of the insecticides CPF and CPF-methyl (https://ec.europa.eu/food/plants/pesticides/approval-active-substances/renewal-approval/chlorpyrifos-chlorpyrifos-methyl_en (accessed on 21 September 2021)). Despite this, these results remain of interest because, according to their persistence observed for other POPs and banned pesticides, CPF will continue to be detected in the environment for several years, similar to endosulfan [63]. In addition, these findings may be valid for other lipophilic and semi-volatile toxicants that may be found in remote areas, such as the synthetic fragrances or the polycyclic aromatic hydrocarbons [7,64]. This study emphasises the importance of considering effects on behaviour (e.g., swimming speed) in early detection of the adverse effects of any stressor in toxicology evaluations and the incipient need to use wild alpine species for the evaluation of natural ecosystems to increase the ecological realism of ex situ experiments. However, a major limitation is that working on ecotoxicological non-model species, without standard experimental guidelines, and which are difficult to rear in the laboratory and are at a risk of extinction, is quite challenging [10].

Future research is needed to detect the long-term and/or indirect effects of organic pollutants, under continuous and prolonged exposure, on different levels of biological organisation to further increase ecological realism of ecotoxicological studies. ‘Omics’ technologies, as adapted from previous ecotoxicology protocols (e.g., [65]), could be employed to further study the physiological effects of these pollutants.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/w13243618/s1>, Table S1: Effect of ethanol on survival, distance and speed in control groups, Table S2: Mean survival, distance and speed of *Diamesa zernyi* larvae exposed at 2 °C to three ethanol concentrations, Table S3: Mean survival, distance and speed of *Diamesa zernyi* larvae exposed to 2, 8 and 11 °C in solution with four different chlorpyrifos concentrations (Conc = 0, 1.1, 11, 110 ng L⁻¹), after 24, 48 and 72 h. Video S1: Effects of CPF on *Diamesa zernyi* swimming behaviour.

Author Contributions: Conceptualisation, V.L. and S.V.; methodology, V.L. and S.V.; software, V.D.N.; formal analysis, V.D.N.; investigation, V.D.N.; resources, V.L.; data curation, V.L. and V.D.N.; writing—original draft preparation, V.L.; writing—review and editing, V.L. and S.V.; visualization, V.D.N.; supervision, V.L. and S.V.; project administration, V.L.; funding acquisition, V.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available because of jointed property with the foundation that financed the project.

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Conflicts of Interest: The authors declare no conflict of interest. The founder CARITRO had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

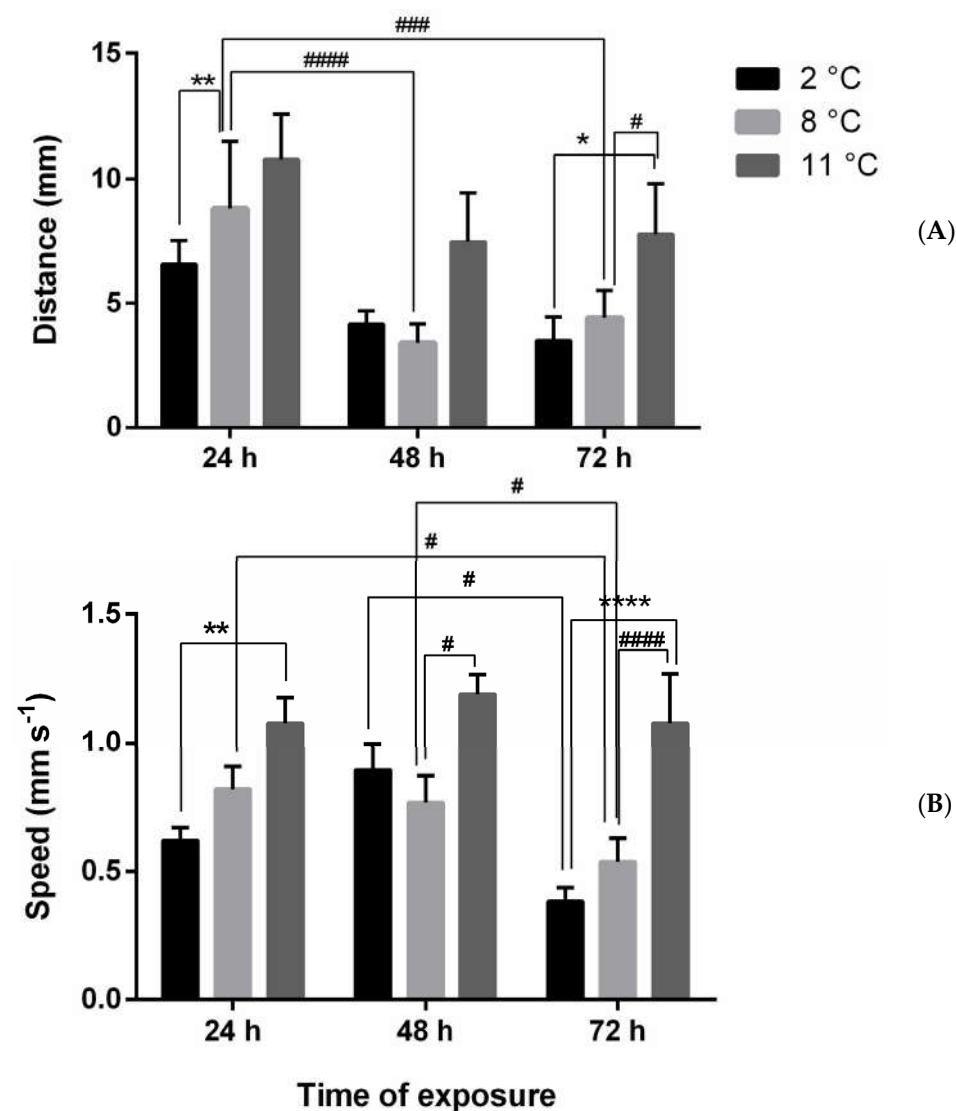


Figure A1. Distance (mm) (A) and speed (mm s⁻¹) (B) of larvae under thermal stress (8 and 11 °C) after 24, 48, and 72 h. Asterisks = significant differences from the control group * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. Significant differences among samples exposed to different concentrations # $p < 0.05$; ### $p < 0.001$; #### $p < 0.0001$.

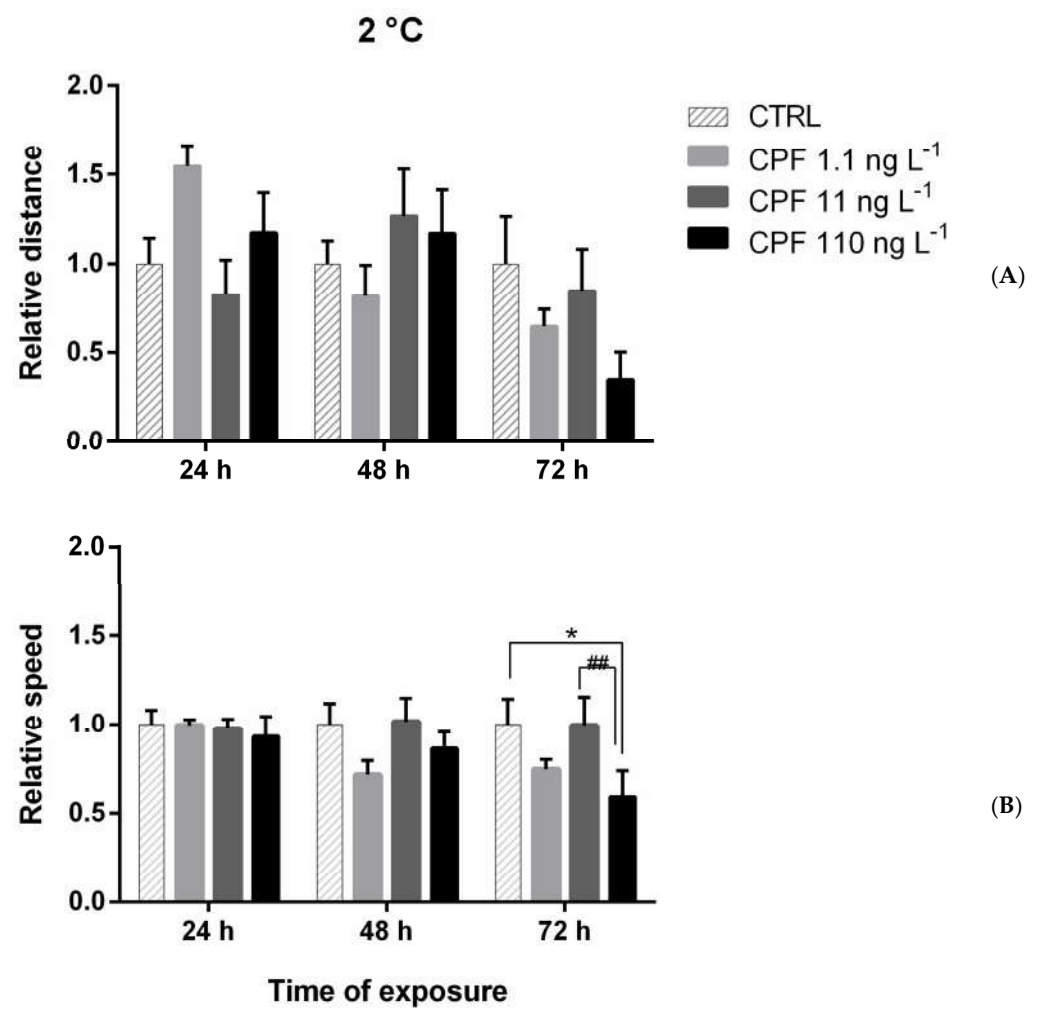


Figure A2. Relative distance (A) and speed (B) of larvae under chemical stress (at 2 °C) after 24, 48, and 72 h of exposure to chlorpyrifos (CPF = 1.1, 11 and 110 ng L⁻¹). Distance values were normalized on the CTRL set equal to 1. Data are Asterisks = significant differences from the control group * $p < 0.05$. Significant differences among samples exposed to different concentrations ^{##} $p < 0.01$.

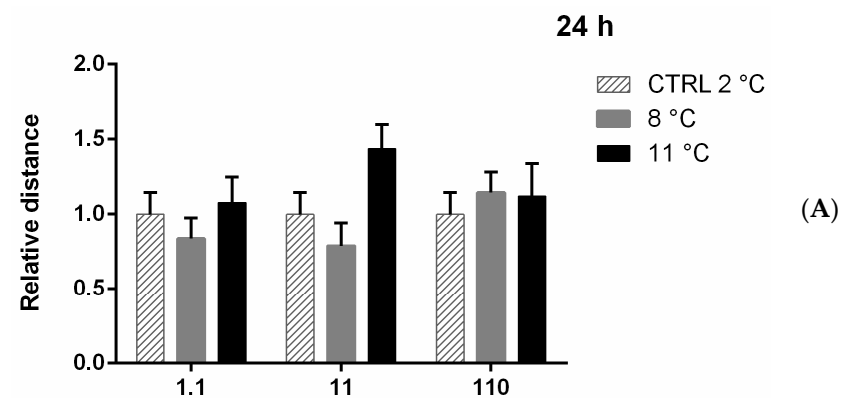


Figure A3. Cont.

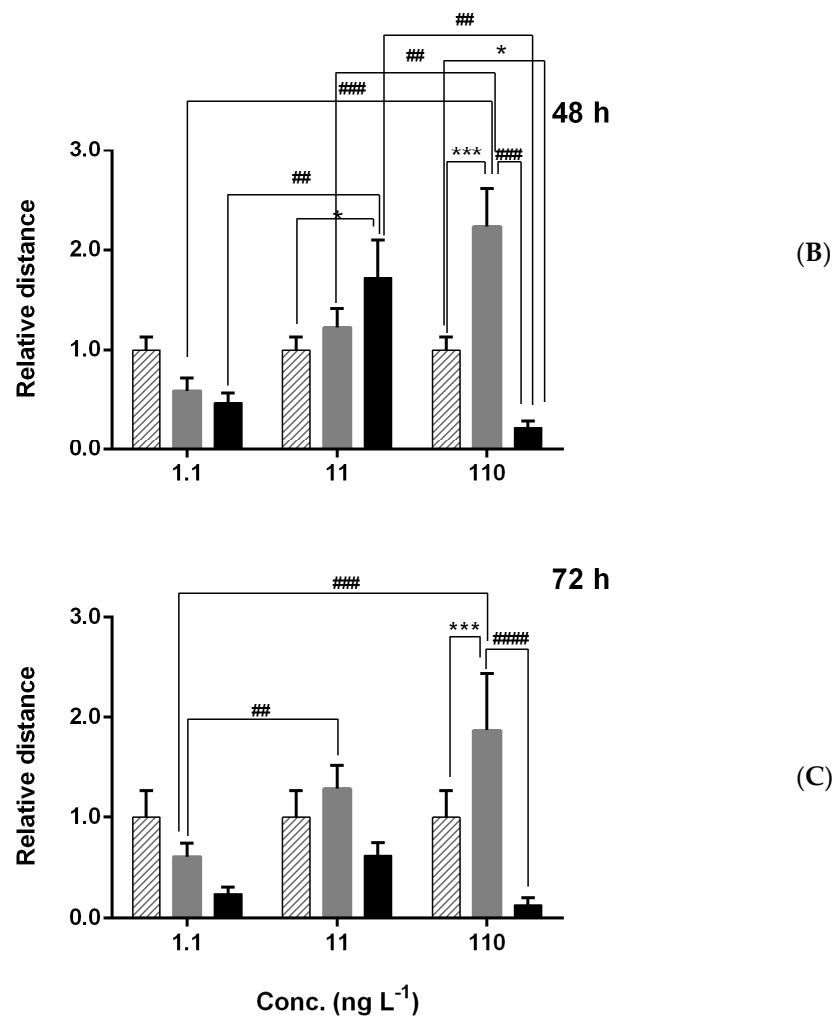


Figure A3. Relative distance of larvae under combined thermal stress (8 and 11 °C) and chemical stress with chlorpyrifos (CPF = 1.1, 11 and 110 ng L⁻¹) after 24 h (A), 48 h (B), and 72 h (C). Asterisks = significant differences from the control group set equal to 1. * $p < 0.05$; *** $p < 0.001$. Significant differences among samples exposed to different concentrations ## $p < 0.01$; ### $p < 0.001$; #### $p < 0.0001$.

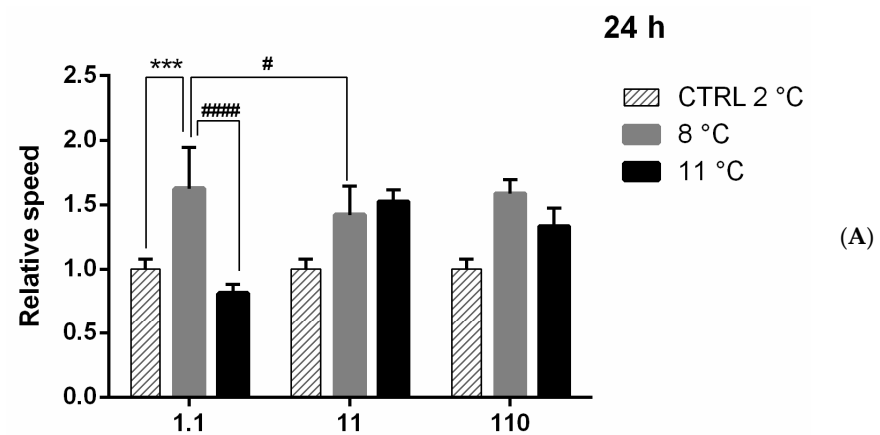


Figure A4. Cont.

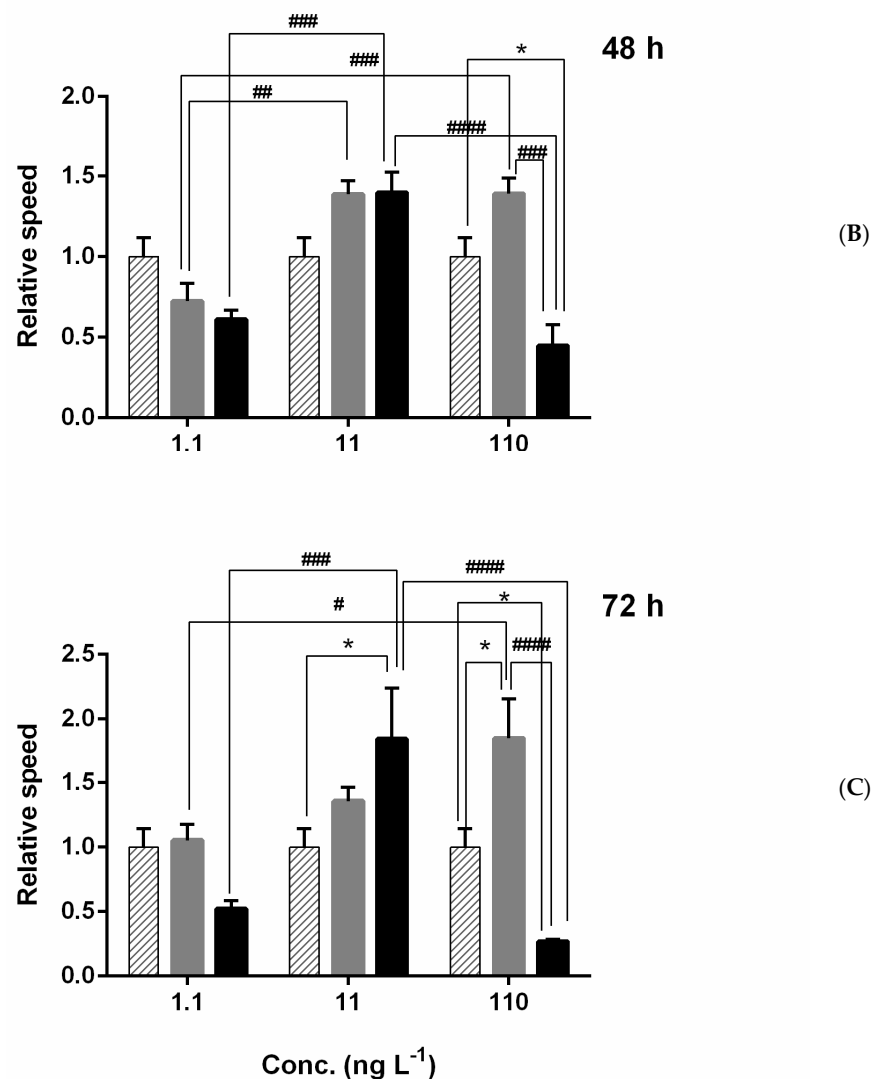


Figure A4. Relative speed of larvae under combined thermal stress (8 and 11 °C) and chemical stress with chlorpyrifos (CPF = 1.1, 11, and 110 ng L⁻¹) after 24 h (A), 48 h (B), and 72 h (C). Asterisks = significant differences from the control group set equal to 1. * $p < 0.05$; *** $p < 0.001$. Significant differences among samples exposed to different concentrations # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$; #### $p < 0.0001$.

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