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Influence of Temperature on the Toxic Effects of Carbamazepine on the Copepod *Tigriopus fulvus*: A Transgenerational Full Life Cycle Study

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Abstract: Coastal areas are increasingly exposed to global warming and emerging contaminants from anthropogenic activities; however, the interactive effects of these stress factors in shaping the offspring's vulnerability to them are poorly understood. The present study aimed to assess the influence of temperature on the toxicity of the pharmaceutical carbamazepine (CBZ) in the parental (F0) and in the first (F1) generation of *Tigriopus fulvus*, through a full life cycle study, measuring several biological parameters. At control temperature (20 °C), exposure to CBZ significantly inhibited larval development, especially in the F1 generation. In contrast, under warmer conditions (27 °C), even after exposure to CBZ, the development was stimulated, proving that temperature was the main factor influencing it. As regards the other investigated life traits (body length, sex ratio, and fecundity), both temperature and generation modulated toxic effects of CBZ, which is evidenced by the onset of higher alterations in F1 co-exposed copepods. Our findings suggest that temperature and contaminants could increase the long-term vulnerability to stressors of *T. fulvus*, potentially affecting the population structure over multiple generations of exposure.

Keywords: global warming; pharmaceuticals; multiple stressors; multiple generations; chronic toxicity

Citation: Parlapiano, I.; Prato, E.; Denti, G.; Biandolino, F. Influence of Temperature on the Toxic Effects of Carbamazepine on the Copepod *Tigriopus fulvus*: A Transgenerational Full Life Cycle Study. *Water* **2024**, *16*, 2051. https://doi.org/10.3390/w16142051

Academic Editor: Heiko L. Schoenfuss

Received: 15 May 2024
Revised: 15 July 2024
Accepted: 17 July 2024
Published: 19 July 2024

1. Introduction

Pharmaceuticals over the last two decades have been receiving increasing attention from the scientific community due to their ubiquitous presence in all environmental compartments [1]. The continuous detection of such compounds in water bodies and the evidence of detrimental biological effects related to exposure resulted in their designation as contaminants of emerging concern (CECs); however, routine monitoring programs, at the moment, do not include them, but may be eligible for future legislation depending on their ecotoxicity [2].

Pharmaceuticals include very complex molecules with different physicochemical properties. Even the most degradable ones are considered persistent or pseudo-persistent compounds because they are constantly discharged into the aquatic environment [3], leading to a scenario of pseudo-chronic exposure to non-target organisms. Considering that pharmaceuticals are capable of being active in small doses, they may exert adverse long-term effects on non-target aquatic organisms systematically far from target organisms, through molecular and metabolic pathways evolutionarily conserved, even at very low concentrations [4].

In marine ecosystems, despite the high dilution factor, pharmaceutical compounds can be found at levels between a few ng/L up to hundreds of µg/L [5,6], posing a growing risk for coastal zones [7].

In addition to anthropogenic pollution, coastal ecosystems are subjected to physico-chemical stressors related to climate change more pronounced compared to the offshore ones [8]. It is estimated that by the end of the XXI century, the surface temperature in certain

coastal zones of the Mediterranean Sea will increase by 4 °C, compared to the 0.6–2 °C predicted in the global ocean [9,10], and there will be at least one long-lasting marine heat wave event every year, up to three months longer, about 4 times more intense and 42 times more severe than current events [10].

The vulnerability of shallow and intertidal coastal habitats to these extreme events may lead to an increase in thermal stress in these environments.

There is a growing amount of research revealing that factors related to global warming can modulate the bioavailability and the effects of several pollutants in coastal environments [11]; anyway, the possible interactions between these co-stressors are still unclear [12,13]. Since ectothermic aquatic organisms are susceptible to changes in temperature because of their biology [14,15], global ocean warming is one of the most common stressors that may affect biological processes in marine species [16,17]; in addition, high temperatures cause a wide variety of contaminants to become more toxic [18]. A rise in temperature, for instance, can alter the transport and the chemical behavior of a substance, and at the same time increase the metabolic rate of organisms and the transport of toxic compounds through food items and cellular membranes, likely resulting in a higher intake of them and consequently a higher toxicity to organisms [19,20]. Therefore, the temperature may affect the traditional ecotoxicological risk assessment of chemicals generally based on the responses obtained with standard toxicity tests under constant and favorable experimental conditions.

To evaluate the influence of increased temperatures on the possible alteration of toxicity of pharmaceuticals, we selected one of the most consumed in the world: the anti-epileptic agent carbamazepine (CBZ) [21]. Because CBZ is lipophilic, resistant to photodegradation and biodegradation, it is also minimally removed in modern multi-stage sewage treatment processes, has a long half-life [22,23], is poorly degraded, and remains in the environment for a long time, leading to its widespread occurrence in the water column up to tens of µg/L and in the tissues of aquatic invertebrates up to 280 ng/g (d.w.) [24–26]. For these reasons, CBZ has been proposed as a marker for anthropogenic contamination [27]. Furthermore, the potential interactions of co-occurring stressors on marine organisms were recently confirmed for pharmaceuticals including CBZ [28–31]. Many studies have demonstrated that CBZ at environmental concentrations affects several physiological, behavioral, and reproductive processes in a variety of non-target aquatic organisms including fish and invertebrates, with significant deleterious effects [32–37]; thus, the European legislation on the classification and labeling of chemicals (92/32/EEC) classified CBZ as "R52/53 Harmful to aquatic organisms and may cause long-term adverse effects in the aquatic environment". As regards marine crustaceans, a recent study has demonstrated that CBZ significantly affected the expression of biomarkers of exposure of the crab *Carcinus maenas*, [38] and inhibited molting and growth of the shrimp *Eriocheir sinensis* [39]; anyway, very limited information is available on the chronic effects of CBZ on full life cycle traits of marine crustaceans.

In a recent study [40], the chronic toxicity of CBZ was assessed in a broad range of environmental concentrations under standard test conditions on the marine meiobenthic copepod *Tigriopus fulvus* (Fisher, 1860). Due to its biological features (small size, short life cycle, high fecundity, distinct post-embryonic stages, sexual dimorphism), *T. fulvus* represents an ideal marine test organism for water quality assessment [40–47]. This copepod is widely distributed along the Mediterranean coasts in shallow supratidal rocky pools; therefore, it is a species subjected to large environmental fluctuations that favor its high adaptability and tolerance [48], so it can be used in ecotoxicological studies under a wide range of environmental conditions. As a generalist feeder, it is an important energy link in marine food webs, and it plays an important role in transferring aquatic pollutants across the food chains [49]. Several studies demonstrated that species belonging to the genus *Tigriopus* are also suitable for use in chronic tests through a multi-generational approach [50–52].

It is becoming increasingly evident that environmental conditions, such as warming and/or contaminants experienced by parents, may influence the performance of multiple generations of offspring in a positive or negative way [53–56]; however, studies on the interactions of co-stressors on successive generations are still scarce. Therefore, the objective of this study was to measure the effects of CBZ on the survival, development, growth, sex ratio, and reproduction across two generations of *T. fulvus* under control temperature test conditions (i.e., 20 °C) and warmer conditions (i.e., 27 °C), at a temperature 4 °C above the surface maximum average value registered in summer over a thirty-year period in the northwestern Mediterranean Sea in *T. fulvus* habitat [10]. Extreme heat wave events with a peak of 27 °C have been observed along the coast in recent years and are known to cause mass mortality events in marine invertebrates [57].

The life traits investigated are key parameters for copepod population recruitment and structure. Our initial tested hypotheses were as follows. (1) The highest temperature increases the direct effects of CBZ on *T. fulvus* with respect to the optimal temperature; (2) the parental exposure to CBZ and temperature shape offspring vulnerability to these stressors, resulting in either enhanced toxicity or resistance in subsequent generations.

2. Materials and Methods

2.1. Test Organisms and Chemicals

T. fulvus used in all experiments came from laboratory cultures established for several months in the CNR Water Research Institute of Taranto, Italy, according to the conditions indicated by the UNICHIM 2396:2014 method [58].

A stock solution of carbamazepine (CBZ) (100 mg/L) (Sigma-Aldrich, Saint Louis, MO, USA, CAS 298–46-4, purity > 97%) was prepared in HPLC-grade methanol for analysis (Sigma-Aldrich, Milano, Italy, CAS 32213-M purity $\geq$ 99.8%) and stored in amber vials at 4 °C until use. Stock solution was diluted in 0.22 µm-filtered artificial seawater (ASW) Instant Ocean® (IO) just before testing, adding the microalgae *Tetraselmis suecica* (10^5 cells/mL) as food source, to obtain working solutions, ensuring that methanol was first completely evaporated. Working solutions were renewed (>80% of the volume) every 48 h. Since CBZ is a very stable compound in aquatic environments, with a half-life of over 100 days [59], any slight changes in the concentration of the solutions during tests were considered negligible.

2.2. Test Procedures

According to previous studies [40,42,46], to start the parental-generation test (F0) a total of 72–78 newly hatched nauplii (<24 h) per treatment was randomly selected and distributed in six replicates into 12 multiwell plates (Nest Biotech Co., Ltd, Wuxi, China). Each replicate/well contained 12–13 nauplii in a volume of 4 mL of test solution. Plates were incubated under the same conditions as the stock culture (16 h light/8 h dark photoperiod, salinity of 38 PSU $\pm$ 1, dissolved oxygen saturation > 80%, pH of 8 $\pm$ 0.3) at two distinctive temperatures (20 °C control and 27 °C).

Copepods were exposed for 28 days to 10 µg/L of CBZ; the tested concentration was chosen based on the results of the previous life cycle experiment mentioned above, in which it was observed that most of the investigated endpoints were significant at 10 µg/L of CBZ [40].

Plates were observed every 24 h under a stereomicroscope with a scattering light Zeiss Stemi SV 11 (Jena, Germany) to determine the developmental stage and calculate the time required for nauplii to become copepodites (naupliar phase duration) and the time required for nauplii to reach the adult stage (i.e., females with ovigerous sac). After all copepods matured, survival (%) and sex ratio (female/male) were assessed. The survival rate was calculated as mean percentage of living nauplii reaching the copepodite and the adult stages at maturity. Moreover, at the end of the experimental period, the body length of male and female specimens was determined under an inverted microscope Zeiss Axiovert S100 (Jena, Germany) equipped with a camera Leica MC170HD with a 10× magnification

using LAS X Leica Microsystem CMS GmbH software (Wetslar, Germany). To measure the fecundity (offspring production), six females with egg sac per concentration were individually transferred to a new 12-well culture plate in a volume of 2 mL of fresh solution per well. Plates were inspected daily to count the number of nauplii per female, the number of broods per female, and the unhatched broods per female, which is defined as the broods unable to produce viable offspring (i.e., aborted egg sacs), and to calculate the time required for the offspring release (hatching time).

Working solutions were changed every 24 h and nauplii removed under the stereomicroscope. The first-generation test (F1) started with nauplii produced by F0 females with the second or third brood; experimental conditions were identical to the F0 generation test. Each generation test was carried out three times.

2.3. Statistical Data Processing

Data were presented as means ± standard deviation (SD). The interactions between the three factors (CBZ × Temperature × Generation) on the dependent variables were not tested because the assumptions for performing the three-way ANOVA were violated.

For this reason, separate tests were applied on the F0 and the F1 generations and to compare the two generations.

Data were tested for normality and variance homogeneity in each generation with Shapiro–Wilk and Levene's tests, respectively. If both assumptions were met, data were examined by analysis of variance (one-way ANOVA), and the Tukey's post hoc test was used to find significant differences ($p < 0.05$) among treatments. If normality and/or homogeneity of variance failed, data were log-transformed. When data did not conform to the ANOVA assumptions, even after transformation, the non-parametric Kruskal–Wallis test followed by the Dunn's post hoc test was run. Specifically, one-way ANOVA was used on untransformed data of survival, length of males and females, hatching time, F0 nauplii/Fov, and on log-transformed data of F1 nauplii/Fov; development, hatching time, brood/Fov, number of nauplii/brood, and unhatched brood were analyzed with the Kruskal–Wallis test.

Since the F1 offspring derived directly from the F0 females (and so generations were dependent), the non-parametric Friedman test for repeated measures followed by the Wilcoxon post hoc test was applied to evaluate the transgenerational effects on variable responses caused by exposure to CBZ at the two temperatures. The two different generations, being dependent, were considered as being a single group from which the repeated measures were taken.

All statistical analyses were performed using Past3 software (version 1.0).

3. Results

3.1. Survival

In both generation F0 and F1, no significant differences were observed in the survival in all treatments ($p > 0.05$); nauplii became copepodites and adults with survivorship > 95%.

3.2. Effects in the F0 Generation

At 20 °C, naupliar phase duration and age of ovigerous females were slightly prolonged in CBZ treatments compared to the control, although differences were significant only for naupliar phase duration. In particular, the time required for nauplii to become copepodites was 5.46 ± 0.50 days in the control and 5.66 ± 0.56 days in the CBZ group, while the time for the appearance of ovigerous females was 14.11 ± 0.68 and 14.50 ± 0.71 days, respectively. On the contrary, at 27 °C, copepod development was significantly accelerated; in fact, the production of copepodites and adult females occurred around the 4th and the 11th days, respectively, both in the control group and the CBZ group (Figure 1A).

Sex ratio (i.e., number of females/number of males) was influenced only by temperature; indeed, this parameter increased at the higher temperature in both treatments

compared to the control temperature, although significantly only in copepods exposed to CBZ.

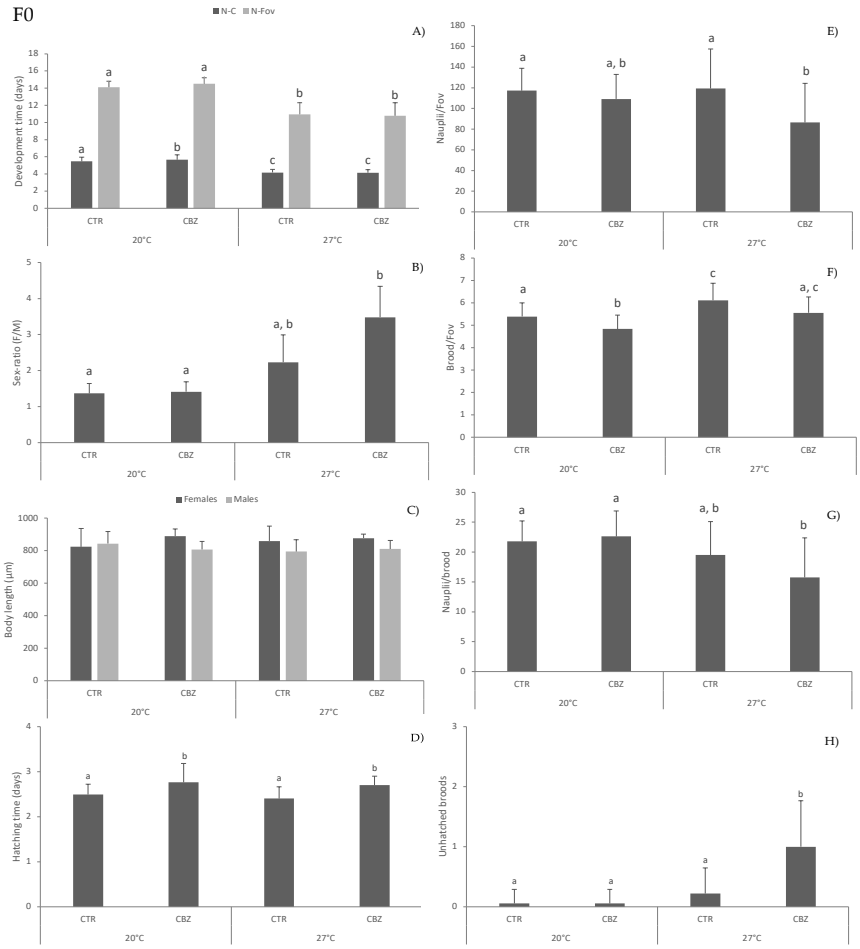

Figure 1. Effect of carbamazepine (CBZ) on life traits of *Tigriopus fulvus* in the F0 generation at 20 °C and 27 °C: (**A**) development time from naupliar to copepodite stage (N-C) and from naupliar to adult stage (i.e., ovigerous female) (N-Fov); (**B**) sex ratio (F/M); (**C**) body length of males and females; (**D**) hatching time; (**E**) number of nauplii per female; (**F**) number of broods per female; (**G**) number of nauplii per brood; (**H**) unhatched broods. Bars with different letters indicate significant differences between treatments and temperatures ($p < 0.05$).

No differences in the body length of males and females exposed to CBZ were observed in the F0 generation with respect to the control at both temperatures (Figure 1C).

Hatching time was influenced only by CBZ, resulting in it being significantly prolonged both at 20 °C and at 27 °C. Hatching occurred after about 2.5 and 2.8 days in the controls and in the CBZ groups, respectively, at both tested temperatures (Figure 1D).

CBZ exposure at 27 °C negatively affected the fecundity; indeed, the mean total number of nauplii per female at the end of the experiments in the control was 119.3 ± 38.3, while in the CBZ group, it was significantly reduced by 27% to 86.4 ± 37.9 (Figure 1E).

The number of broods was 5.4 ± 0.6 and 6.1 ± 0.8 in the control groups and 4.8 ± 0.6 and 5.6 ± 0.7 in the CBZ groups, at 20 °C and 27 °C, respectively, highlighting a reduction in exposed groups, although significant only at 20 °C. The statistical analysis also evidenced a significant increase in the control and the CBZ group at 27 °C, compared to both treatments at the control temperature (Figure 1F).

A reduction of the mean number of nauplii per brood occurred only at 27 °C, where there were fewer nauplii per brood in both treatments (19.5 ± 5.6 in the control and 15.8 ± 6.6 in the CBZ group) with respect to the control temperature (21.8 ± 3.4 in the

control and 22.6 ± 4.2 in the CBZ group), although the differences were significant only in the CBZ group (Figure 1G).

Temperature and CBZ exerted a significant effect on the number of aborted sacs. The mean number of unhatched broods per female, in fact, was significantly higher only in copepods exposed to CBZ at 27 °C with respect to the other treatments (Figure 1H).

3.3. Effects in the F1 Generation

As regards naupliar phase duration, in the F1 generation, it was observed that the same trend occurred as in the F0 generation, although the magnitude of the CBZ effect was more pronounced in the offspring; indeed, nauplii in the CBZ group at 20 °C became copepodites after 6.3 ± 0.9 days.

Differently from the F0 generation, a significantly slower maturation of females was also detected in the CBZ group at 20 °C in comparison to the control group; in fact, the mean age of ovigerous females was 15.4 ± 1.1 days (Figure 2A).

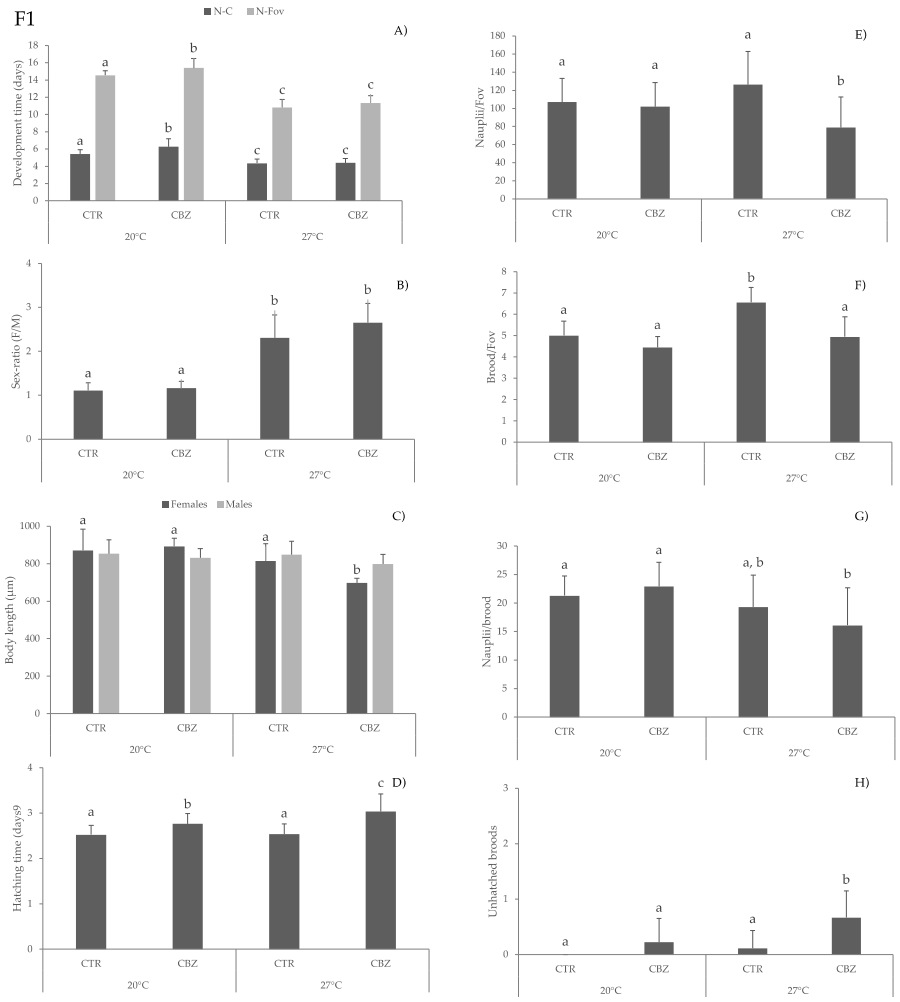

Figure 2. Effect of carbamazepine (CBZ) on life traits of *Tigriopus fulvus* in the F1 generation at 20 °C and 27 °C: (**A**) development time from naupliar to copepodite stage (N-C) and from naupliar to adult stage (i.e., ovigerous female) (N-Fov); (**B**) sex ratio (F/M); (**C**) body length of males and females; (**D**) hatching time; (**E**) number of nauplii per female; (**F**) number of broods per female; (**G**) number of nauplii per brood; (**H**) unhatched broods. Bars with different letters indicate significant differences between treatments and temperatures ($p < 0.05$).

Temperature strongly influenced the sex ratio in the F1 generation as well as in the F0 one. The sex ratio significantly increased at the higher temperature in both treatments compared to the control temperature (Figure 2B).

Differently from the F0 generation, at 27 °C, the length of females was significantly reduced in the CBZ group, measuring 698.0 ± 25 µm, compared to the control group, measuring 814.7 ± 91.6 µm, and compared to 20 °C. Indeed, at the control temperature, females measured 870.7 ± 113 and 891.8 ± 43.7 µm in the control in the CBZ treatment, respectively (Figure 2C). Finally, as regards the number of unhatched broods, the same pattern was observed as in the F0 generation.

Unlike the F0 generation, both temperature and CBZ influenced hatching time. In fact, at 27 °C, in the F1 copepods exposed to CBZ, a significant increase was observed in the duration of hatching time (3.0 ± 0.4 days) compared to the copepods exposed to CBZ at 20 °C (Figure 2D).

Like in the F0 generation, CBZ exposure negatively influenced the mean number of nauplii per female, although the observed reduction (38%) at 27 °C was stronger compared to the control group (Figure 2E).

As regards the number of broods, unlike the F0 generation, responses at 27 °C were more pronounced, resulting in a reduction of 25% in the CBZ treatment with respect to the control, and an increase of about 30% in the control with respect to the control at 20 °C (Figure 2F).

3.4. Transgenerational Effects

The non-parametric test for repeated measures evidenced that at the control temperature, nauplii of the F1 generation exposed to CBZ became copepodites with a significant delay of about 0.6 days with respect to the copepods exposed to CBZ of the F0 generation. At 27 °C, both in the control group and in the CBZ group, naupliar phase duration was significantly prolonged with respect to the F0 generation (Table 1).

Table 1. Results of the different tested endpoints (mean ± SD) for *Tigriopus fulvus* exposed to carbamazepine (CBZ) in the F0 and F1 generations at 20 °C and at 27 °C.

Endpoint	**20 °C**			
Generation	***F0***		***F1***	
CBZ (µg L^{-1})	0	10		10
N-C * (days)	5.46 ^{a} ± 0.56	5.66 ^{b} ± 0.28	5.46 ^{a} ± 0.56	5.66 ^{c} ± 0.28
N-Fov * (days)	14.11 ^{a} ± 0.68	14.50 ^{a} ± 0.71	14.56 ^{a}± 0.51	15.39 ^{b} ± 0.71
Sex ratio (F/M)	1.37 ± 0.27	1.41 ± 0.28	1.11 ± 1.18	1.16 ± 0.16
Female length (µm)	823.65 ± 90.33	889.83 ± 64.35	870.69 ± 113.02	891.82 ± 43.74
Male length (µm)	843.39 ± 97.29	807.10 ± 80.00	853.80 ± 74.53	831.04 ± 49.10
Hatching time (days)	2.49 ^{a} ± 0.23	2.77 ^{b} ± 0.42	2.52 ^{a}± 0.21	2.76 ^{b} ± 0.23
Nauplii/Fov	117.22 ± 21.63	109.11 ± 23.92	107.06 ± 26.05	101.83 ± 26.91
Nauplii/brood	21.78 ± 3.45	22.62 ± 4.24	21.30 ± 3.35	22.91 ± 5.29
Brood/Fov	5.39 ^{a} ± 0.61	4.83 ^{b,c} ± 0.62	5.00 ^{a,b} ± 0.69	4.44 ^{c} ± 0.51
Unhatched broods	0.06 ± 0.23	0.06 ± 0.23	0.00 ± 0.00	0.22 ± 0.43
	27 °C			
Generation	***F0***		***F1***	
CBZ (µg L^{-1})	0	10	0	10
N-C * (days)	4.16 ^{a} ± 0.37	4.14 ^{a} ± 0.37	4.35 ^{b} ± 0.37	4.40 ^{b} ± 0.37
N-Fov * (days)	10.94 ± 1.35	10.78 ± 1.52	10.83 ± 0.92	11.33 ± 0.84
Sex ratio (F/M)	2.23 ± 0.76	3.48 ± 0.86	2.31 ± 0.52	2.65 ± 0.44
Female length (µm)	859.43 ^{a} ± 67.00	876.83 ^{a} ± 30.53	814.73 ^{a} ± 91.61	698.04 ^{b} ± 25.02
Male length (µm)	795.13 ± 102.55	810.97 ± 108.99	848.40 ± 71.40	798.53 ± 51.47
Hatching time (days)	2.41 ^{a} ± 0.26	2.70 ^{b} ± 0.20	2.54 ^{a} ± 0.23	3.03 ^{b} ± 0.39
Nauplii/Fov *	119.33 ^{a} ± 38.27	86.44 ^{b} ± 37.85	126.28 ^{a} ± 36.82	78.78 ^{b} ± 33.94
Nauplii/brood	19.51 ± 5.60	15.76 ± 6.61	19.31 ± 5.46	16.10 ± 6.00
Brood/Fov *	6.11 ^{a} ± 0.76	5.56 ^{b} ± 0.70	6.56 ^{a} ± 0.70	4.94 ^{b} ± 0.94
Unhatched broods	0.22 ^{a} ± 0.43	1.00 ^{b} ± 0.77	0.11 ^{a} ± 0.32	0.67 ^{a} ± 0.48

Notes: * Fov: ovigerous female; N-C: development time from naupliar to copepodite stage; N-Fov: development time from naupliar to adult stage (i.e., ovigerous female). Different letters indicate significant differences between both treatments and generations ($p < 0.05$).

In the F1 generation, at 20 °C, the time for the appearance of ovigerous females exposed to CBZ was significantly prolonged by about 0.9 days compared to the F0 generation

(Table 1), reflecting the delay observed in the duration of the naupliar phase. A slight delay of 0.5 days was also observed in females exposed to CBZ at 27 °C with respect to the F0 generation, although this was not significant (Table 1). Furthermore, at 27 °C, the body length of F1 females exposed to CBZ was significantly reduced by 22% compared to the F0 females exposed to CBZ.

4. Discussion

Most previous studies investigating the combined effects of contaminants and temperature as co-stressors have centered on short-term experiments not considering the relevance of chronic exposure, especially over multiple generations [60]. CBZ is known to exert deleterious effects on aquatic non-target species, affecting development and reproduction [39,40,61–63], and enhanced toxicity has been observed when combined with marine heatwaves in *Mytilus galloprovincialis* [28].

Concerning crustaceans, existing knowledge on the combined effects of CBZ and temperature is limited to the freshwater shrimp *Atyaephyra desmarestii*, in which CBZ, under increasing temperature conditions, at a concentration of 13.8 µg/L, may produce respiratory deficiencies causing alterations in its behavior [64].

In the present study, based on previous results obtained on the parental generation of the marine copepod *T. fulvus* at the control temperature (i.e., 20 °C) [40], we examined the toxic effects of CBZ on development and reproduction over two generations at two different temperatures. Generally, such results demonstrated that, except for the survival and the size of males, CBZ exposure and temperature—alone or in combination—negatively affected all the sub-lethal investigated life cycle traits of *T. fulvus*, with stronger effects in the F1 generation.

The transition from the naupliar stage to the copepodite stage and from naupliar stage to adult stage (i.e., females with ovigerous sac) was confirmed to be a sensitive endpoint [40,42,46]. At the control temperature, in F0 copepods exposed to CBZ, it was observed that a slight slowdown of development occurred compared to the control. Since CBZ is a potential endocrine disruptor, it could have affected the metamorphosis process that is necessary for growth and development. In this regard, Chen et al. [39,62] showed that exposure to 3 and 10 µg/L of CBZ can inhibit the molting process of *Daphnia similis* and *Eriocheir sinensis*, respectively, by interfering with the activity of chitinolytic enzymes and the signaling of molting hormone, causing a slowdown of development. In the present study, the delay observed in copepods exposed to CBZ in the F1 generation was significantly more pronounced with respect to the F0; nauplii became copepodites and adults with a delay of about 0.6 and 0.9 days, respectively. Considering that CBZ is a relative lipophilic (K_{ow} = 2.2) and bioaccumulative compound [65–67], the increased retardation in maturation observed in the F1 generation with respect to the F0 generation could have been caused by a longer time of exposure to the pharmaceutical. Several studies on aquatic invertebrates revealed that pharmaceuticals bioaccumulated in the exposed organisms may be transferred through the mother to eggs and deposited in the offspring, leading to a transgenerational transfer of toxicity [68]. As regards the results observed under seawater warming conditions (i.e., 27 °C) for these life traits, temperature strongly affected the development of *T. fulvus* compared to the CBZ, appearing to be the primary factor that influenced it. Warming also significantly shortened the development time of *T. fulvus* after CBZ exposure, especially in the parental generation. This could be explained considering that marine copepods tend to develop faster to the reproductive age with the increase in temperature [69–72]. In accordance with our results, Li et al. [73] showed that the development time of the congener *T. japonicus* decreases with high temperature. In the F1 females exposed to CBZ, the acceleration of maturation was less noticeable, probably due to an enhanced absorption of CBZ in the offspring.

As regards sex ratio, according to previous studies on *T. fulvus* [40,42,46], a trend toward more females per male, which is quite common for harpacticoid copepods [74–76], was observed in all treatments. In general, the existing literature shows a relatively high

variability of sex ratio in crustaceans under warmer conditions. For example, in the calanoid *Paracartia grani* reared under warmer conditions for several generations, sex ratio was close to 1, showing to be not influenced by temperature [77]; on the contrary, in the harpacticoid *T. californicus*, a sex ratio shift (more males per female) was observed with the increasing temperatures, even after several generations [78]. Our results showed that temperature alone affected sex ratio, which increased from a minimum of 1.1 at 20 °C to a maximum of 3.5 at 27 °C. Similarly, Koch et al. [79] showed an increasing ratio of females per male from 0.7 to 4.0 with increasing temperatures in the harpacticoid *Nitokra spinites*. The sex determination in response to environmental factors could be an adaptative mechanism which needs to be further investigated, since a highly biased sex ratio may influence the size and the reproductive output of a population [80].

Another life trait influenced by temperature was the body length of females. Female size at maturity could be an important parameter influencing fecundity of copepods, as observed by Horne et al. [81]. The results obtained evidenced that CBZ enhanced the negative influence of warming on growth. This was particularly evident in the F1 co-exposed females, which showed a size reduction by 14% compared to the control females at 27 °C and by 22% compared to the CBZ-exposed females at 20 °C. Our results confirm that under warmer conditions, copepods develop faster, achieving smaller sizes at maturity [82–84], with consequences for their role on trophic web and community structure. For example, the increase in mean temperature recorded in an estuary on the Atlantic coast of the USA caused a decrease in body size of two species of the genus *Acartia*, the disappearance of two species of large copepods, and an increase in the proportion in the community of the small copepod *Oithona* sp., leading to an alteration in community composition [85]. In addition, smaller organisms are more sensitive to chemicals, which in turn can cause a decrease in body size through a variety of mechanisms [82]. Alterations in development and growth may be accompanied by impairment of other physiological traits, such as fertility.

In the present study, the fecundity-related traits of *T. fulvus* exposed to CBZ (i.e., number of broods/females, number of nauplii/broods and number of nauplii/females) were significantly reduced compared to the control in both generations.

The toxicity of CBZ was modulated by temperature, as evidenced by the onset of greater alterations caused by combined stressors rather than single stressors, but also by generation. Overall, after 28 days' exposure, the most severe effects on reproduction were observed in F1 co-exposed females, in which the mean number of nauplii and broods decreased by 38% and 25%, respectively, compared to the control females, evidencing that the combined effect of both stressors may lead to greater toxicity on subsequent generations.

At environmental levels, CBZ has been proven to cause oxidative status alterations, lipid peroxidation, immune system impairment, and genotoxic damage in marine invertebrates [28,36,67]. This may diminish the investment of energy for reproduction, because of the increased energy costs for defence, detoxification, and repair mechanisms, as is well documented in cladoceran freshwater crustaceans [86,87].

Previous studies showed that the reproductive output of *Daphnia similis* [62], *D. magna* [61], *D. pulex* [88], and *Ceriodaphnia dubia* [89] significantly decreased after chronic exposure to CBZ at low concentrations ranging from 0.03 µg/L to 200 µg/L. Chen et al. [62] demonstrated that CBZ may act as an endocrine disrupter in *D. similis*, interfering with the endocrine system, which regulates various physiological functions in crustaceans, such as development, growth, sex ratio, and reproduction. In accordance with our results, in a multigenerational study, Nkoom et al. [63] found that exposure to 2 and 10 µg/L of CBZ significantly inhibited growth, molting frequency, and reproduction, and induced significant behavioral changes in *D. magna*, in the parental as well as the first generation, with relatively greater effects in the first one. Furthermore, the simultaneous reduction in the expression of molting, as well as reproductive and antioxidant-related gene expression, suggests that this pharmaceutical may cause oxidative stress and act as an endocrine disruptor. In another recent study, He et al. [90] reported that 5 µg/L of CBZ altered the

expression of genes related to reproduction and toxic metabolism in two generations of *D. magna*, but that the transcriptional changes at the gene level were not fully translated into physiological performance in the F0 generation, while they were translated into a significant reduction in offspring in the F1 generation. According to these results, our findings also suggest that exposure to CBZ could act as an endocrine disruptor in *T. fulvus* and could also determine genetic alterations, as evidenced by the occurrence of greater effects in the first generation.

Hatching time was significantly extended in copepods exposed to CBZ with respect to the control, both at 20 °C and 27 °C in both generations, with a maximum increase of 0.5 days in F1 co-exposed females, but no differences occurred between the two generations. The prolonged hatching time indirectly affected fecundity, resulting in fewer broods. Finally, copepods aborted their egg sacs in response to both stressors. Specifically, F0 co-exposed females aborted the highest number of sacs, while in the F1 co-exposed females, there was a lower disturbance of temperature, suggesting a greater incidence of the pharmaceutical.

Overall, results reported here showed a greater incidence of the pharmaceutical on reproductive outcome, which could be the consequence of its greater bioaccumulation through the maternal transfer in the offspring [68].

At the same time, our results provide clear evidence of the capability of temperature to increase *T. fulvus* sensitivity to CBZ. Indeed, although, under warmer conditions, copepods began to reproduce earlier, the major inhibition of fecundity occurred in co-exposed females. This could be explained considering that a higher temperature increases the transport and uptake of contaminants across cell membranes, in response to an increased metabolic rate, which can result in the enhanced sensitivity of exposed organisms [91].

Recent studies on aquatic invertebrates highlighted the role of parental exposure to warming and contaminants in shaping the susceptibility of offspring to stressors. In the mosquito *Culex pipiens*, the interaction between warming and the insecticide chlorpyrifos was synergistic in both generations, despite the increased tolerance to chlorpyrifos in the offspring [92]; in the freshwater water flea *Moina dubia*, the antagonistic interaction between warming and lead (Pb) increased gradually across 10 exposed generations [93]. Similarly to our findings, in the planktonic copepods *Pseudodiaptomus annandalei* and *P. incisus*, parental exposure to Cu and a simulated marine heatwave resulted in lower performance of offspring [56,94]. Finally, warming interacted with nickel (Ni) and increased its multigenerational toxicity, reducing survival and reproduction of the benthic copepod *T. japonicus* [52].

5. Conclusions

The present study investigated the chronic toxicological effects of CBZ combined with temperature on the copepod *T. fulvus* over two generations. In addition to the confirmed capability of CBZ to affect development and reproduction of *T. fulvus* [40], the results obtained evidenced that temperature may modulate the sensitivity of such copepods to CBZ. Indeed, while at 20 °C, the exposure to CBZ inhibited the development of *T. fulvus*, at 27 °C, and also after CBZ exposure, copepods developed significantly faster, evidencing that the effect of the pharmaceutical was mitigated by the influence of temperature on this life trait. In contrast, growth and reproduction inhibition was greater under the warmer condition, especially in the first generation, as evidenced by co-exposed females, in which the greatest reduction in body size and fecundity was observed. The reduction in the reproductive fitness could compromise copepod population structure across generations. Although ocean warming and pharmaceutical exposure are major threats to aquatic ecosystems, and temperature can strongly alter contaminants' toxicity, few studies have focused on how their combined effects can be transmitted to the next generation. The findings reported here highlight the need to consider maternal effects in environmental risk assessment for multiple stressors, as the influence of pollutants during single-generation exposure may be underestimated.

Author Contributions: Conceptualization, I.P., E.P. and F.B.; Methodology, F.B.; Formal analysis, G.D.; Investigation, I.P.; Data curation, I.P. and G.D.; Writing—original draft, I.P. and F.B.; Writing—review & editing, E.P.; Supervision, E.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Conflicts of Interest: The authors declare no conflict of interest.

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