Long-term exposure of the red cherry shrimp Neocaridina davidi to diclofenac: impact on survival, growth, and reproductive potential

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Abstract

The current study was aimed at studying the long-term effects of diclofenac on the freshwater shrimp *Neocaridina davidi*, concerning survival, somatic growth, and reproduction. To this study, both ovigerous females and males of this species were exposed for 63 d to 0 (control), 0.1, or 1 mg/L of diclofenac. At the highest concentration, significant mortality was detected, and the somatic growth of females was significantly decreased. The percentage of females with a second spawn, observable from day 45, significantly increased at 1 mg/L, while the time between spawns was significantly reduced at both concentrations assayed. However, the gonadal analysis made at the end of the assay in the surviving females showed a significantly lower proportion of advanced oocytes in females exposed to 1 mg/L, as compared to control. Concerning hatching, the percentage of ovigerous females that could have successful hatching was reduced at 1 mg/L of diclofenac, especially for the first spawn. For the second spawn, the number of juveniles hatched from females exposed to 1 mg/L was reduced compared to control; these juveniles also showed a significantly higher incidence of morphological abnormalities, such as hydropsy and underdeveloped appendages. Taken together, these results showed that even when diclofenac was able to produce earlier spawns, the reproductive output of each spawn was reduced. No differences in the spermatophore structure were seen in the distal *vas deferens* of surviving males.

Introduction

Among the emerging pollutants, pharmaceuticals for human and veterinary usage represent a major concern. These drugs are discharged into the aquatic environment through both urban and rural effluents. The so-called non-steroidal anti-inflammatory drugs (NSAIDs) occupy the first place in environmental presence, compared to other pharmaceuticals (Vazquez-Carrillo et al. 2020; Santos et al. 2010). Diclofenac is one of NSAIDS most used worldwide. Unfortunately, this kind of pharmaceutical is not efficiently cleaned by sewage treatment plants. For instance, only 45.6% (on average) of diclofenac was reported to be cleaned in Portuguese treatment plants (Pereira et al. 2015). Although diclofenac levels slightly higher than 1 μg/L have been reported in Argentine rivers, levels near or even higher than 10 μg/L of diclofenac were found in other countries of South America, such as Colombia and Venezuela (Valdez-Carrillo et al. 2020).

The main mode of action of NSAIDs is the inhibition of the cyclo-oxygenase enzyme, which catalyzes the synthesis of prostaglandins from arachidonic acid. The precise role of prostaglandins in the ovarian development of crustaceans seems to be species-specific; particularly in both shrimps and prawns, the reports made show decreased levels of prostaglandins (mainly the $PGF_{2\alpha}$ variant) with the advance of ovarian growth (Alfaro-Montoya 2015; Wimuttisuk et al. 2013). Recent reviews have characterized diclofenac as causing a wide variety of effects in the aquatic fauna, such as oxidative stress, and impairment of growth and osmoregulation, although studies in crustacean reproduction are scarce (Parolini et al. 2020; Sathishkumar et al. 2020).

The red cherry shrimp, *Neocaridina davidi* (Bouvier, 1904) (Atyidae) is a tiny freshwater decapod species with fast growth, maturation, and highly abbreviated development. Females are easily identified by their yellow/green ovaries, which become visible through the carapace in shrimps weighing more than 40 mg (Tropea et al. 2015), achieved around 45 days post-hatching (Vazquez et al. 2017). Ovarian re-maturation takes place a few days after hatching or even during egg incubation (Barbier 2010; Tropea and López Greco 2015). Fertilized eggs remain attached to female pleopods during the incubation period, which lasts approximately 15 days at 27 ± 1°C (Tropea et al. 2015) and the number of hatched juveniles per spawning ranges from 14 to 80 (Tropea et al. 2015, 2022; Sganga and López Greco 2019). At hatching, juveniles have remaining lipid droplets in the hepatopancreas defining them as lecithotrophic juveniles (Sganga and López Greco 2020). Females spawn several times throughout a life span of 1.5 years under culture conditions (Barbier 2010) maintaining a good brood quantity at least during six consecutive spawns (Tropea and López Greco 2015; Marciano et al. 2018).

According to Parolini et al. (2020), freshwater invertebrates are excellent model species to assess NSAIDs toxicity. In the case of *N. davidi*, the above-mentioned life-cycle characteristics turn this species into a good decapod crustacean model to evaluate if exposure to different contaminants may exert toxic effects on growth and reproduction, especially across successive generations (López Greco et al. 2021).

This research was aimed at characterizing the deleterious effects of diclofenac on survival, growth, and reproductive potential through two consecutive spawning events in *N.davidi*, identifying the concentrations at which such effects take place.

Material And Methods

Animals

Both females and males of *N. davidi* used in the present study were born from the reproductive stock in the laboratory; parentals were obtained from a local dealer. This stock was maintained for several weeks in plastic aquaria filled with 8 L of dechlorinated tap water under continuous aeration (pH 7.5, hardness 80 mg/L as $CaCO_3$ equivalents) and at a constant temperature of 27 ± 1°C. Photoperiod was held at 14L:10D. Each aquarium was provided with Java moss (*Vesicularia* sp.) as shelter. Shrimps were fed daily *ad libitum* on commercial-balanced food for tropical fish (Tetracolor®, Germany, 47.5% of crude protein), as in previous studies on the species (Tropea and López Greco 2015; Tropea et al. 2015, 2018, 2022). The reproductive stock was daily inspected to detect the presence of ovigerous females and mature males with spermatophores in the *vasa deferentia*. Once detected, one ovigerous female together with two mature males (needed to induce ovarian re-maturation in females, Tropea et al. 2018) were assigned to a glass aquarium containing 1.5 L of dechlorinated tap water. Mature males ready to mate were recognized by the presence of spermatophores in the distal *vasa deferentia* through the translucent carapace (Tomas et al. 2019). Each of these aquaria was considered a replicate and was randomly assigned to every treatment. Water from all aquaria was completely replaced three times a week. Feeding

regime, water quality, temperature, and photoperiod held throughout the assay were the same used for the stock maintenance, as mentioned.

Diclofenac administration

Based on preliminary range finding tests, shrimps were exposed to the following treatments: control (clean water, N = 22 replicates), diclofenac sodium salt (DCF) 0.1 mg/L (N = 22 replicates) and DCF 1 mg/L (N = 22 replicates). The mean weight of ovigerous females used was 111.13 ± 0.01 mg, while males weighed 37.27 ± 0.01 mg. Concentrated stock solutions of diclofenac sodium salt (Sigma Co., USA) were prepared weekly by dissolving the appropriate amount of diclofenac sodium in distilled water. Small aliquots were taken from these stock solutions, for diluting in the corresponding test recipient, to achieve the desired concentration of the pharmaceutical. In order to validate the nominal concentration assayed, water samples (15 mL) were taken at 0 and 72 h, i.e., the maximum period for water replacement in all test containers. Nominal concentrations were validated by means of HPLC, in 10 mL water samples taken from two replicates at 0 and 72 h. Briefly, solid-phase extraction of water samples was made with C18 cartridges using methanol as the desorption solvent. All extracts were filtered at 0.22 µm and injected into an HPLC chromatograph (Alliance 2695, Waters®), coupled to an MS/MS tandem (Quattro Premier XE, Waters®), equipped with an electrospray ionization interface. A CSH C18 column with a methanol/water gradient was used, supplemented with formic acid at 0.1%. Analytical guidelines recommended by the European Community (SANTE/11813/2017) were followed. As a quality control of the water matrix used in the assay, isotopic 5D-atrazine and 10D-acetochlor standards were added to known concentrations of the studied analytes in saline water, as tracers for checking the technical reproducibility, and then processed in parallel with the exposure solution samples. A 20% of these samples were also enriched with the same isotopic compounds as internal standards, to estimate the recovery percentages.

Endpoints

The experiment comprised 63 ± 3 days, in order to allow embryo development until hatching in a first reproductive cycle, and a subsequent ovarian re-maturation, followed by mating, embryo development, and second hatching. It is important to remark that full re-maturation takes place approximately 45 d after a previous spawning, according to previous studies (Tropea and López Greco 2015; Marciano et al. 2018). Shrimps were daily inspected to record deaths, molts, egg loss, hatching juveniles, and re-maturation ovary progress. Once juveniles hatched, all of them were counted (actual fecundity, according to Steachey and Sommers 1995), weighted (precision 0.1mg) and the maximum juvenile length on a subsample of 10 individuals was measured from the tip of the rostrum up to the telson, under a stereoscopic microscope Carl Zeiss, based on Tropea and López Greco (2015). The specific growth rate of adults was also calculated as (ln(Fw)-ln(Iw))*100/t, where t is the time elapsed between the final (Fw) and initial (Iw) wet body weight; for males, Iw was measured at the beginning of the assay, as a mean value per aquarium, while Iw of females was measured just after hatching. In all cases, Fw was measured at the end of the assay.

A lecithotrophic index (area of the hepatopancreas occupied by lipid droplets in mm^2/total area of cephalothorax in mm^2) was also quantified in recently hatched juveniles (as in Sganga and López Greco 2019) by means of the FIJI program, version 2.3.051 (Schindelin et al. 2012). After this, all juveniles were fixed in formalin 5% and then transferred to ethanol 10% to analyze morphological abnormalities based on Pantaleão et al. (2017). At the end of the experimental period, both females and males were cold-anesthetized for 5 min and weighted (precision 0.1mg). Finally, they were fixed in Bouin solution for 24 h at 4 ºC, following the histological procedure for the species based on Tomas et al. (2019). Histological sections were analyzed to recognize possible morphological abnormalities in male distal *vasa deferentia*. In females, fixed ovaries were dissected, all oocytes were counted under a stereoscopic microscope (Carl Zeiss), photographed, and the oocyte´s area was measured by using the FIJI program. According to their size and coloration, oocytes were classified as initial (pre-vitellogenic) or advanced (vitellogenic).

Statistical analyses

Data analyses were performed according to Zuur et al. (2009) using the R Studio version 4.2.0 (R Core Team, 2022). Before statistical analyses, normality, homogeneity of variances, and/or data dispersion were checked. Mortality, and the percentages (as proportions) of both initial and advanced oocytes per female, females with hatching eggs, females with a second spawn, and abnormal juveniles hatched per female were analyzed by means of a generalized linear model with binomial distribution. The number of hatched juveniles, the time elapsed between spawns, and the incubation time were analyzed using a generalized linear model with Poisson distribution. Specific growth rate (SGR) of animals, oocyte area, mean length, and the lecithotrophic index of juveniles hatched per female were tested by one-way ANOVA (treatment as factor). The significance level was always set at 0.05. In all cases, multiple comparisons were made using Tukey's test (Sokal and Rohlf 1981).

Results

Concentrations of diclofenac measured in the experimental test containers are detailed in Table 1. No substantial differences with nominal concentrations were observed. The mortality percentage in replicates, for every treatment, is shown in Fig. 1; a significant ($p < 0.05$) increase in mortality was only observed at DCF 1 mg/L, compared to control (Fig. 1). Concerning SGR, a significant ($p < 0.05$) reduction was observed for surviving females exposed to DCF 1 mg/L, and no significant differences ($p > 0.05$) were detected for males (Fig. 2).

Table 1
Nominal versus measured concentrations of diclofenac. The maximum variation was estimated by measuring just after water replacement (0 h) and just before it (72 h).

Nominal diclofenac concentration (mg/L)	Measured concentration (mg/L)		
	0 h	72 h	Overall mean
0	0.009	0.006	0.0075
0.1	0.1577	0.1595	0.1320
	0.1191	0.0917	
1	1.1663	0.8534	1.0730
	1.4171	0.8550	

Table 2 also shows the reproductive parameters measured during the assay. The proportion of females with a second spawn was estimated from the number of females alive at day 45 of the assay, the date at which full ovarian re-maturation was expected, and in fact, a second spawn just began to be observed. An increment in the percentage of these females was detected as the diclofenac concentration increased, being significant ($p < 0.05$) with respect to control at 1 mg/L (Table 2). Besides, the time between spawns was significantly lower at both diclofenac concentrations, compared to the control group (Table 2).

Table 2
Number and percentages of surviving *Neocaridina davidi* shrimps, females with a second spawn, and females with hatching eggs with only one (first) or two (second) spawn, for each experimental group. The time between spawns is also detailed. Asterisks indicate significant ($p < 0.05$) differences with the respective control.

Number (N) or percentage	Diclofenac concentration (mg/L)		
	0 (control)	0.1	1
Alive males at the end of the assay (N)	37	28	26
Alive females at the end of the assay (N)	14	12	10
Alive females (N) on day 45th (ovarian re-maturation)	19	18	14
Percentage of females with a second spawn (after day 45th)	21.05	50	71.43*
Days elapsed between spawns (N)	47 ± 3	37 ± 2*	37 ± 2*
Percentage of females with hatching eggs - first spawn	90.91	86.36	54.55*
Percentage of females with hatching eggs - second spawn	100	55.56	50

Figure 3A shows the proportion of both initial and advanced oocytes in the ovaries of *N. davidi* females, at the end of the 63-d exposure period. A significant ($p < 0.05$) lower proportion of advanced oocytes were found between the highest concentration and control. Concerning the area of each type of oocyte

(Fig. 3B), no significant differences ($p > 0.05$) were seen between each diclofenac concentration and control. Concerning males, examined at the end of the assay, the exposure to diclofenac caused no structural alterations in the spermatophores within the distal *vas deferens*, compared to the concurrent control. The observed structures were similar to those described in un-exposed shrimps in previous studies (Tomas et al. 2019).

For the first spawn (i.e., ovigerous females at the beginning of the assay), the percentage of females with hatching eggs was significantly ($p < 0.05$) reduced by 36.4% in the group treated with DCF 1 mg/L (Table 2). Although no significant ($p > 0.05$) differences were detected for the second spawn, a reduction in that percentage was observed in both groups exposed to DCF, with respect to control (Table 2). No significant ($p > 0.05$) differences were noted in the incubation time, for any of the spawns (Fig. 4A). For every treatment, the number of juveniles hatched from the second spawn was significantly ($p < 0.05$) lower than the number corresponding to the first spawn; besides, although no significant ($p > 0.05$) differences were detected, a marked decrease in the number of hatched juveniles was observed between both diclofenac concentrations and control, for the second spawn (Fig. 4B)

No significant ($p > 0.05$) differences in size were observed in juveniles from females exposed to diclofenac, compared to controls (Table 3). Concerning the lecithotrophic index (Fig. 5B), no significant ($p < 0.05$) differences between exposed and controls were noted (Table 3). The abnormalities detected in hatched juveniles (shown in Figs. 5C, E) were hydropsy (abnormal hydration, mainly in the cephalothorax, likely caused by disruption of osmoregulatory mechanisms), and underdeveloped appendages (pereiopods, spines, setae, and antennae).

Table 3
Size measurements in hatched juveniles, and percentage of abnormal juveniles per female. Different letters indicate significant ($p < 0.05$) differences among treatments.

Spawn	Treatment	Length (mm)	Lecitotrophic index	% of abnormal juveniles per female
First	Control	3.49 ± 0.03	0.408 ± 0.004	12.09 ± 5.66
	DCF 0.1 mg/L	3.46 ± 0.04	0.410 ± 0.008	13.56 ± 7.04
	DCF 1 mg/L	3.48 ± 0.03	0.411 ± 0.009	18.13 ± 9.12
Second	Control	3.47 ± 0.02^{ab}	0.399 ± 0.006	1.04 ± 1.04^{a}
	DCF 0.1 mg/L	3.53 ± 0.02^{a}	0.397 ± 0.018	0
	DCF 1 mg/L	3.40 ± 0.02^{b}	0.376 ± 0.004	15.64 ± 9.24^{b}

Discussion

During the assay, mortality showed to be significant only at the highest concentration assayed (1 mg/L), reaching 45%. In *Daphnia magna*, the 21-d LC50 of diclofenac was estimated at 2 mg/L (Du et al. 2016); this is comparable with the results obtained in the current study after 63 d of exposing *N. davidi* males to the same compound, taking into account the difference in size between both species. In *Neocaridina denticulata*, similar in size to *N. davidi*, the 96-h LC50 value of ibuprofen, another NSAID of lower toxicity, was 6.07 mg/L (Sung et al. 2014).

Somatic growth, in terms of a decrease in the SGR, was only significantly reduced in females at 1 mg/L. Heckmann et al. (2007) have reported a reduction in the population growth rate of *D. magna* exposed to 20 mg/L of ibuprofen, while diclofenac was able to reduce the energy available for growing of mussels *Mytilus edulis trossulus* after some weeks of exposure to concentrations as low as 0.1 mg/L, also affecting the byssus integrity (Ericson et al. 2010). In juveniles of *Lymnaea stagnalis* freshwater snails, diclofenac inhibited shell growth, as well as feeding behavior (Bouly et al. 2022).

Concerning the reproductive parameters determined in the current study, a significant increase in the proportion of females with a second spawn was observed, at the highest concentration of diclofenac. Besides, at that concentration, the time between spawns was reduced. Taken together, these results are indicating an acceleration of ovarian re-maturation, induced by diclofenac. According to the reports made on other shrimp species, the prostaglandin levels are high at the beginning of ovarian growth (i.e., immature oocytes) to decrease later, as oocytes became mature (Alfaro-Montoya 2015; Wimuttisuk et al. 2013). Based on this temporal variation, disruption of the normal pattern of prostaglandin synthesis caused by diclofenac could cause an acceleration of the ovarian re-maturation. In accordance with this possible effect, Alfaro-Montoya (2015) has reported in the shrimp *Litopenaeus vannamei* a higher rate of females with developing ovaries after 5 weeks of exposure to 0.1 µg/g of ibuprofen. In *D. magna*, the expression of vitellogenin was induced after acute (24 to 96 h) exposure to diclofenac, at concentrations ranging from 0.05 to 5 mg/L (Liu et al. 2017). In the mussel *Mytilus galloprovincialis*, an increase in the synthesis of vitellogenic proteins has been also reported in those exposed to diclofenac and other pharmaceuticals (González Rey et al. 2014). Also in mussels, a significant decrease in prostaglandin-E_2 levels was verified after the acute exposure to 0.1 mg/L of diclofenac (Courant et al. 2018).In addition to the inhibition of prostaglandin synthesis, a possible effect of diclofenac altering other endocrine controls of ovarian growth (e.g., given by sexual steroids) should not be discarded; in this sense, some evidence of endocrine imbalances has been reported in the fish *Astyanax lacustris*, concerning the secretion of gonadotropins (Souza Branco et al. 2021).

Despite the acceleration caused by diclofenac on the ovarian re-maturation, in terms of a high proportion of females with a second spawn and a lower time between spawns, the proportion of mature oocytes at the end of the assay was not increased in the exposed group; on the contrary, a reduction in the proportion of advanced oocytes was noted in the group exposed to 1 mg/L. These results correlated with the decrease in the SGR detected at the highest concentration, suggesting that not only the energy investment for somatic growing is reduced, but also the energy devoted to ovarian growth, which could affect a further spawn. Besides, a reduction in the proportion of mature oocytes would lead to a reduction

in the number of hatched juveniles in the next reproductive cycle. Although not statistically significant, such reduction was seen, especially for the second spawn, potentially due to a longer exposure time. Moreover, it is expected that at times of exposure even longer, a more marked reduction in the reproductive output of each spawn occurred, in terms of a decreased number of both mature oocytes and hatched juveniles. Despite the decrease in the proportion of mature oocytes, no differences were seen between exposed and control animals in the oocyte size, in correlation with the fact that the lecithotrophic index was similar among treatments. These later results would indicate that those juveniles that successfully hatched in the groups exposed to diclofenac would not have been affected concerning the transference of energy reserves from oocytes. Nevertheless, the biochemical quality of the vitellum could have been affected, with a possible impact on juvenile performance (e.g. somatic growth, survival). This matter would need further research.

Several authors have reported some kind of inhibitory effect of NSAIDs on the reproduction of aquatic invertebrates. Hence, Lee et al. (2011) reported a reduction in the number of young per female of *D. magna* after 3-month of exposure to diclofenac. In the same species, Heckmann et al. (2007) reported a 14-d EC50 of 13.4 mg/L of ibuprofen, for the inhibition of reproduction. Induction of the Hsp70 heat shock protein has been also observed in *D. magna* after acute exposure to 30 mg/L of diclofenac (Haap et al. 2008). Bonnefille et al. (2018) have found an impairment of both osmoregulation and reproduction in *M. galloprovincialis* exposed to diclofenac, in correlation with interference on tyrosine and tryptophan metabolism. In addition, oxidative stress was induced in the amphipod *Hyalella azteca* acutely exposed to diclofenac (Oviedo-Gómez et al. 2010), while both genetic and cellular damage, attributable to oxidative stress, has been reported in the freshwater bivalve *Dreissena polymorpha* acutely exposed to a concentration as low as 0.2 µg/L (Parolini et al. 2011).

The deleterious effect of diclofenac, either due to oxidative stress or any other mode of action, could also impact the embryos developing into spawned eggs. In fact, at the highest diclofenac concentration, a clear reduction in the percentage of females with hatching eggs was observed for both spawns, being statistically significant for the first one. In principle, this is talking about embryo mortality leading to egg abortion. However, an impairment of the egg care behavior of ovigerous females, which would lead to the same result, should not be discarded. A significant reduction in egg production was reported in *D. magna* exposed to 0.5 mg/L of diclofenac (Du et al. 2016). In *M. galloprovincialis* exposed to several NSAIDs, including diclofenac, several genotoxic and cellular effects were noted after 60 days of exposure to 2.5 µg/L (Mezzelani et al. 2018). In the medaka fish exposed to diclofenac at concentrations ranging from 0.001 to 10 mg/L, a significant decrease in hatching success was seen (Lee et al. 2011)

Moreover, a significant incidence of morphological abnormalities was detected in nearly 20% of juveniles hatched per female, in the 1 mg/L diclofenac group. The fact that this relatively high incidence of abnormalities was detected only for the second spawn is suggesting that some accumulative effect of diclofenac could have taken place, progressively impacting the ovary, to be later transferred to embryos. However, such abnormalities were relatively unspecific, since they have been reported in other crustacean species exposed to several kinds of pollutants (Rodríguez et al. 2007), and even in controls to a lower

extent. Hydropsy (an increase in volume due to abnormal hydration) is likely related to a disruption in osmoregulation, an effect attributable to a variety of pollutants, including diclofenac (Bonnefille et al. 2018 in *M. galloprovincialis*, Eades et al. 2010 in *Carcinus maenas*). Undeveloped setae and spines could also be related to osmoregulatory imbalances, since a high hemolymphatic pressure is needed for the eversion of such structures (Rodríguez et al. 2007).

However, a possible genotoxic effect of diclofenac should not be discarded. As mentioned before, this drug is able to produce oxidative stress and genetic damage in crustaceans (Parolini et al. 2020). For instance, diclofenac induced DNA methylation in adults of the marine copepod *Gladioferens pectinatus*, leading to abnormalities in embryos (Guyon et al. 2018). In *M. galloprovincialis*, the *in vitro* exposure to 1 and 10 µg/L of diclofenac after fertilization, produces shell malformations, by affecting genetic expression (Balbi et al. 2018). Diclofenac produced up to 40% of abnormalities in zebrafish embryos exposed to 0.0125 to 1.25 mg/L, and 100% at 12.5 mg/L (Ribeiro et al. 2015). Although some effect of diclofenac was seen on the sperm motility of echinoderms (Zanuri et al. 2017), no evidence of disruption in either the sperm production or spermatophore formation of *N. davidi* has been found in the current study.

Conclusions

We conclude that under long-term exposure at concentrations ranging between 0.1 an 1 mg/L, diclofenac is able to produce several deleterious effects in the freshwater shrimp *Neocaridina davidi*, causing higher mortality and reducing both the somatic and gonadal growth of females. Although diclofenac shortened the time between spawns, it produced a lower proportion of re-matured oocytes, reduced hatching, and a higher incidence of abnormal progeny

Declarations

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Competing interests

All authors declare they have no financial interests.

Author Contributions

Laura S. López Greco and Enrique M. Rodríguez contributed equally to supervision and experimental design. Conducting of experiments, data collection, and statistical analysis were performed by Marina Zanitti; Laura S. López Greco also aided in the characterization and management of biological material. Daniel A. Medesani helped with technical issues throughout the experiments. The first draft of the manuscript was written by Enrique M. Rodríguez; comments from all authors were incorporated to achieve the final version.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request

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Figures

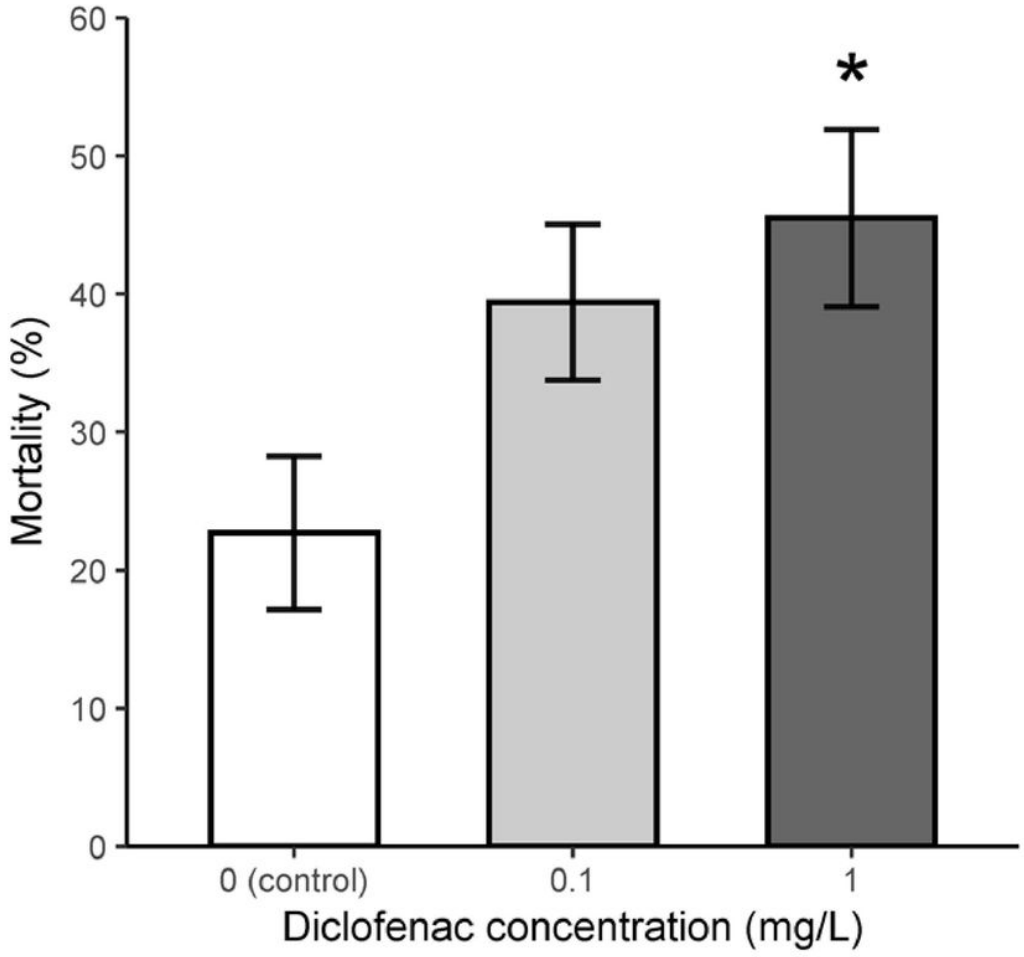

Figure 1

Mortality percentage (mean ± standard error) in replicates (N=22 in each treatment), during the entire assay. Asterisk indicates significant differences ($p<0.05$) with respect to control.

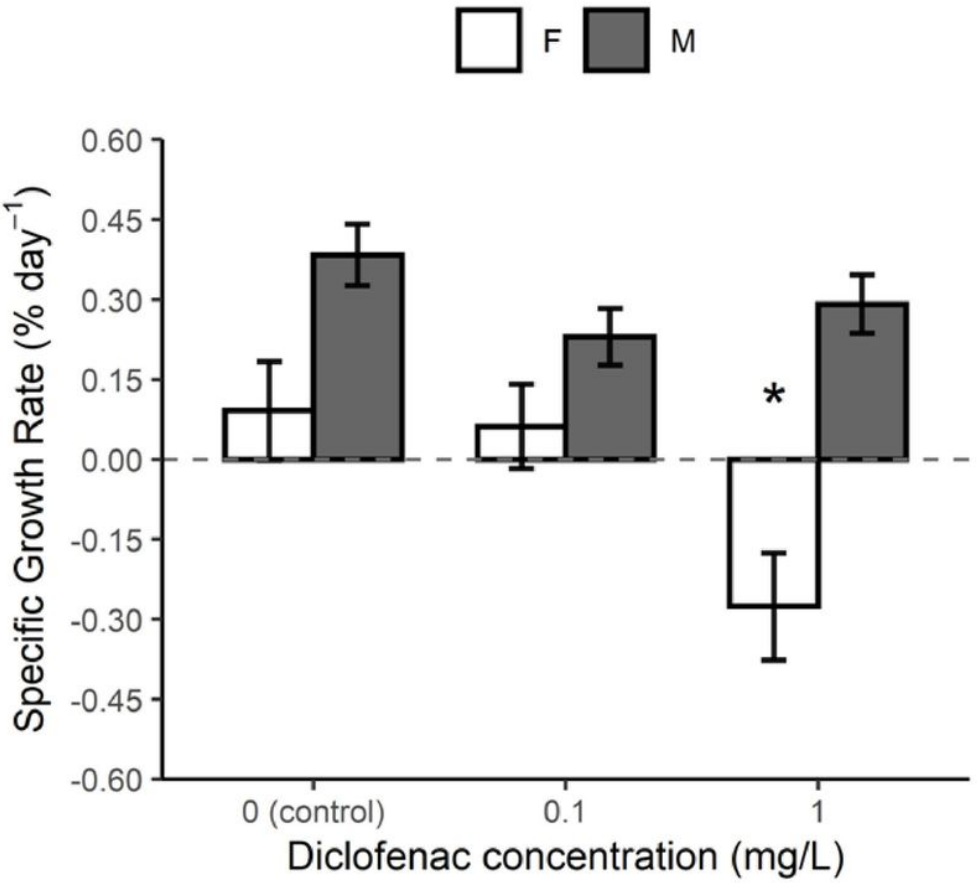

Figure 2

Specific growth rate (% per day) of *N. davidi* females (F) and males (M). Mean values ± standard error are expressed. Asterisk indicates significant differences ($p<0.05$) with respect to control.

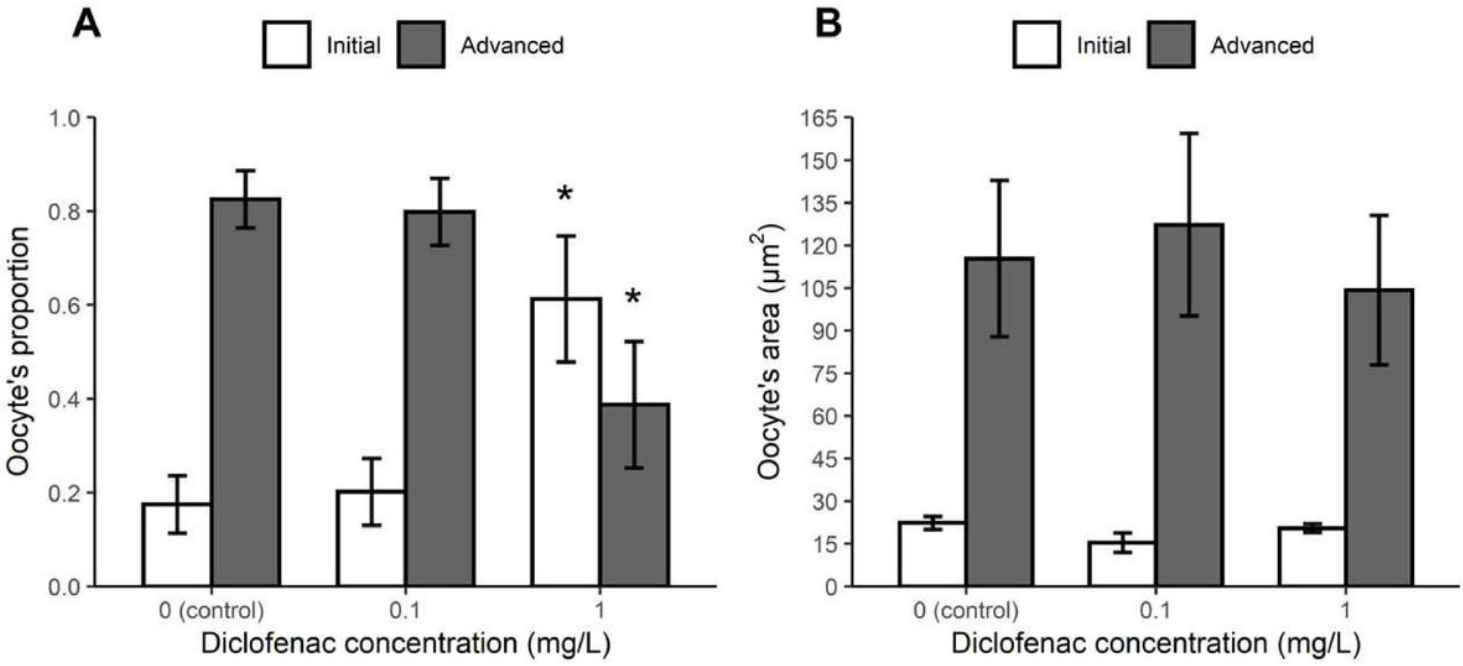

Figure 3

A: proportion of initial and advanced oocytes, and B**: o**ocyte area, for each oocyte type, determined in ovaries of *N. davidi* at the end of the 63 d exposure period. Asterisks indicate significant ($p<0.05$) differences with control. In all cases, mean ± standard error is expressed.

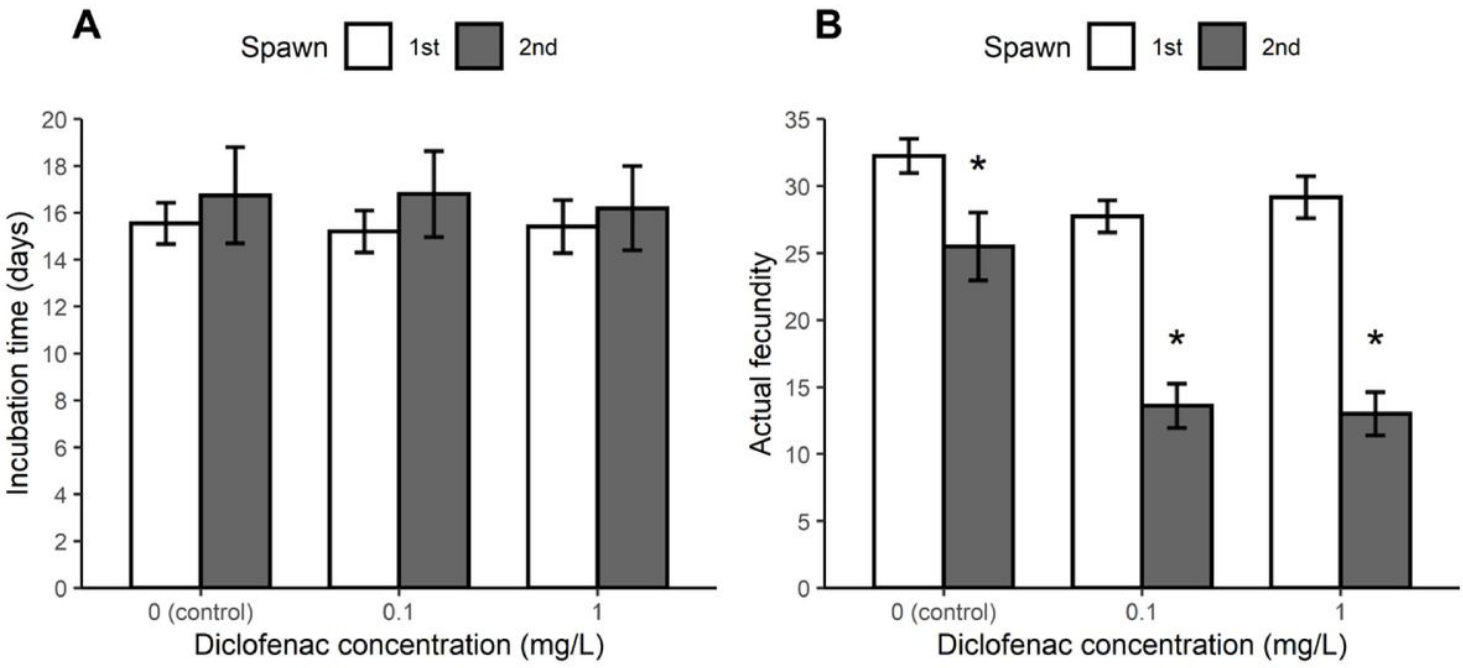

Figure 4

A: incubation time, for every treatment and spawn. B: number of hatched juveniles per female (actual fecundity) for *N. davidi* exposed to DCF. Asterisks indicate significant differences between spawns, for each treatment ($p<0.05$). In all cases, mean ± standard error is expressed.

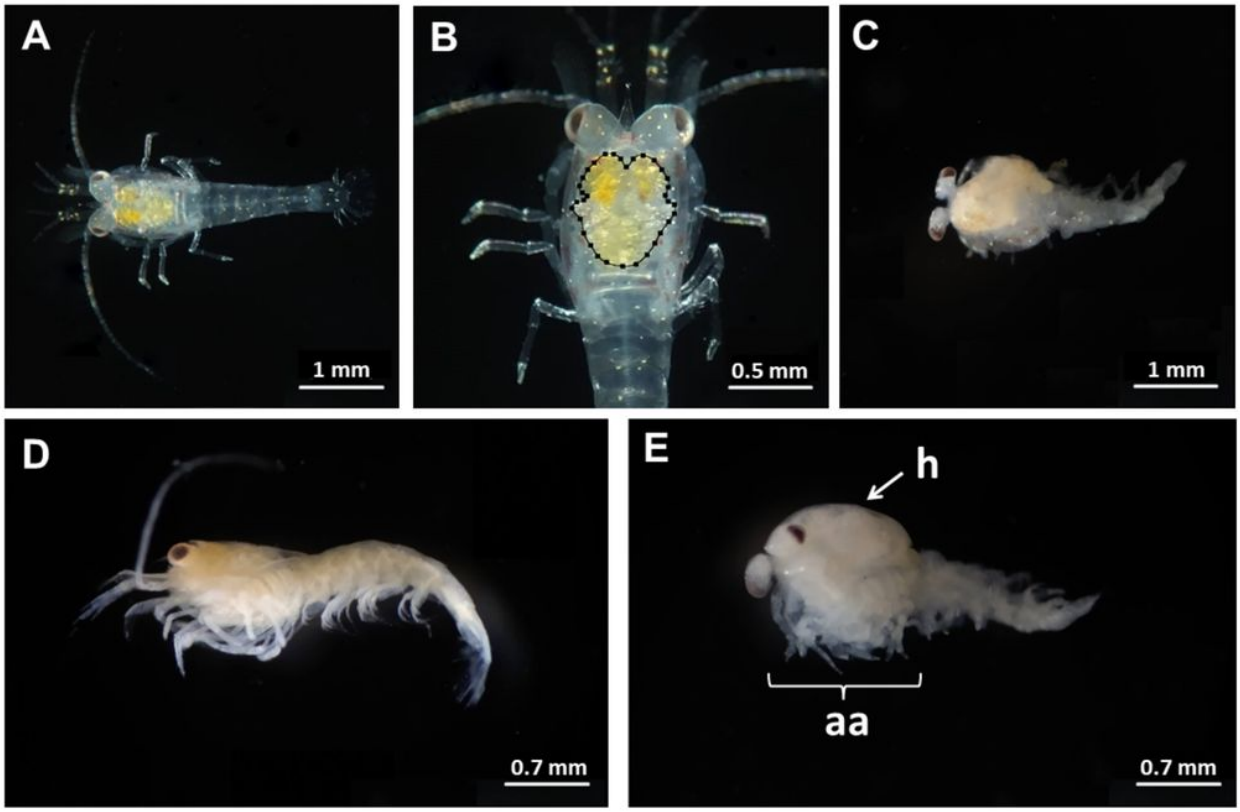

Figure 5

A**:** normal juvenile, hatched from a control female of *Neocaridina davidi*. B: **a**rea of the hepatopancreas occupied by lipid droplets (surrounded by dots, used to calculate the lecithotrophic index, after relativizing it to the carapace area); **C:** abnormal juvenile, hatched from a female exposed to DCF 1 mg/L. Juveniles shown in A, B, and C were photographed while they were alive. D: normal, and E: abnormal juvenile, after fixation; aa: abnormal (underdeveloped) appendages; h: hydropsy.