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RESEARCH ARTICLE

Sublethal effect of the toxic dinoflagellate *Karlodinium veneficum* on early life stages of zebrafish (*Danio rerio*)

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Abstract

Dinoflagellates of the genus *Karlodinium* are ichthyotoxic species that produce toxins including karlotoxins and karmitoxins. Karlotoxins show hemolytic and cytotoxic activities and have been associated with fish mortality. This study evaluated the effect of toxins released into the environment of *Karlodinium veneficum* strain K10 (Ebro Delta, NW Mediterranean) on the early stages of *Danio rerio* (zebrafish). Extracts of the supernatant of K10 contained the mono-sulfated KmTx-10, KmTx-11, KmTx-12, KmTx-13, and a di-sulfated form of KmTx-10. Total egg mortality was observed for karlotoxin concentration higher than 2.69 μg L^{-1}. For 1.35 μg L^{-1}, 87% of development anomalies were evidenced (all concentrations were expressed as KmTx-2 equivalent). Larvae of 8 days postfertilization exposed to 1.35 μg L^{-1} presented epithelial damage with 80% of cells in the early apoptotic stage. Our results indicate that supernatants with low concentration of KmTxs produce both lethal and sublethal effects in early fish stages. Moreover, apoptosis was induced at concentrations as low as 0.01 μg L^{-1}. This is of great relevance since detrimental long-term effects due to exposure to low concentrations of these substances could affect wild and cultured fish.

Keywords Karlotoxin KmTx · Ichthyotoxin · Harmful algal bloom · Plankton · Zebrafish bioassay

Introduction

Harmful microalgal blooms of the genus *Karlodinium* are responsible for mortalities of wild and cultured aquatic species worldwide (Deeds et al. 2002; Kempton et al. 2002; Adolf et al. 2007; Place et al. 2012). *Karlodinium* are small marine and estuarine dinoflagellates (~8–12 μm) belonging to the family Kareniaceae and commonly found in coastal aquatic ecosystems (Goshorn et al. 2004; Bachvaroff et al. 2008; Place et al. 2012) where they produce outbreaks regularly (reviewed in Place et al. 2012). Fish kills by *Karlodinium* seems to have increased in the last decades. Recent blooms have been reported in different locations, for instance, Malaysia (*K. australe*; (Lim et al. 2014)), East Asia (*K. digitatum*; (Sakamoto et al. 2021)), USA (*K. veneficum*; (Wolny et al. 2022)) or Angola (*K. veneficum*; (Pitcher and Louw 2021). *Karlodinium* is typically present at relatively low cell abundances (10^2–10^3 cell mL^{-1}) but can form dense blooms (10^4–10^5 cell mL^{-1}) (Deeds et al. 2002, 2006). Exposure to high *Karlodinium*'s cell densities has been shown to elicit a response in fish gills including increased ionic permeability, oedema, hyperplasia, and epithelial necrosis (Deeds et al 2006).

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Some species of the genus *Karlodinium* produce a class of ichthyotoxins called karlotoxins (KmTxs). Several of them have been identified (Takeshita et al. 2000; Van Wagoner et al. 2008, 2010; Place et al. 2012; Waters et al. 2015; Cai et al. 2016; Krock et al. 2017). These are large (molecular weight > 1000 Da), amphipathic compounds whose ecological role could be related to chemical defence against grazing and/or their use for prey acquisition's (Adolf et al. 2007; Waggett et al. 2008; Place et al. 2012). On the other hand, it has been found recently that some species produce additional closely related ichthyotoxic toxins. For instance, *K. armiger* produces karmitoxin (Binzer et al. 2020). In addition, it has been shown that *K. veneficum* toxins have a significant allelopathic effect on the growth of *P. donghaiense* (Wang et al. 2020). In Table 1, the isolated congeners studied to date have been included with their corresponding bioactivities. KmTxs have been characterized by their hemolytic in vivo or in vitro activity and have been claimed to kill fish by damaging their gill epithelia (Deeds et al. 2002, 2006, 2015; Kempton et al. 2002; Bachvaroff et al. 2009). The potency of the KmTx congeners, regarding hemolytic activity, depends greatly on the congener. Moreover, different studies have also shown significant variability. For instance, the range of EC_{50} is 47–5245 ng mL^{-1} for KmTx-1 and sulfo-KmTx-10, respectively. It should be noted that comparisons based on these EC_{50}s must be carefully made since different species and methodology have been used. In addition, these studies were performed using toxin preparations at varying stages of purification, as no reference standards are available for this toxin group. In the case of KmTx-2 (as in others), the observed EC_{50} range for the same hemolytic assay was 368–1768 ng mL^{-1} (erythrocytes of *Oncorhynchus mykiss*). The most studied analogues are KmTx-1 and KmTx-2 (Deeds et al. 2002; Kempton et al. 2002). In vivo studies have been conducted almost exclusively with these two congeners, with KmTx-2–1 being the only exception (Adolf et al. 2007; Mooney et al. 2010). Rapid morphological changes in zebrafish larval epithelia have been evidenced when exposed to high doses of KmTx-2 (4 µg mL^{-1}) presenting mortality within the first 15 min of exposure, and they also manifest in intense cell swelling and epithelial detachment in exposed surfaces (Deeds et al. 2006). However, epithelial damage can be observed at a much lower concentration (EC_{50} = 800 ng mL^{-1}) (Deeds et al. 2006). The specific action mechanisms of KmTxs are not completely known, but it has been suggested that KmTxs could act by forming pores in the cell membranes by binding to membrane lipids. Since different cell lines and animals are sensitive to these toxins, the pore-formation process is probably not initiated by a very specific lipid-binding phenomenon. Notwithstanding, it has been shown that the lytic effect (and self-protection) is dependent on the content or type of sterols in the target membranes (Mooney et al. 2009; Rasmussen et al. 2017). Previous studies showed that KmTx-2 sublethal doses produce an in vitro increase in the permeability of the plasma membrane to certain cations (Na^{2+}, Ca^{2+}, and Mn^{2+}) (Deeds et al. 2015). This prelytic action could initiate an apoptotic-like pathway leading to severe damage in the gill´s epithelia (Rasmussen et al. 2017). The apoptotic process could be regulated by the increase of cations such as calcium and the formation of pores in the plasma membrane. Since some ichthyotoxins have been shown to trigger apoptosis (Qi et al. 2016), the molecular mechanisms underlying the alteration of the osmotic balance, oxidative stress or the loss of membrane's functionality, among others, are relevant for the understanding of the risk of sublethal exposure (Zhang et al. 2018).

Strains of *Karlodinium veneficum* showed high chemical variability regarding KmTxs composition, although in some cases it could be influenced by growth conditions as well (Krock et al. 2017). In the cited work, genetic comparison was carried out for species and strain identification based on ITS and LSU rDNA sequences (Krock et al. 2017). Most of the characterized strains showed higher toxicity in their culture supernatants than in the cell extracts. This would suggest that KmTxs are released to the environment in natural conditions. However, certain amounts of toxins can also be released due to mechanical stress during filtering or centrifugation. Thus, it appears that KmTxs may act as allelochemicals related to ecological strategy (Wang et al. 2020), chemical defence against grazing and/or in prey acquisition (Place et al. 2012). It has been suggested that the length of the lipophilic arm is an important determinant of potency and that sulfation and chlorination could also influence the damage on biological membranes (Place et al. 2012; Adolf et al. 2015). There is little information however on the effects of KmTxs at sublethal concentrations on species that cohabit in the water column, their potential bioaccumulation in these, and on human health (due to consumption of contaminated fish).

Recent investigations have strengthened the utility of early-stages*Danio rerio* in marine microalgae toxicity's evaluation, either to evaluate changes in toxicity induced by different nutrients conditions (*Alexandrium tamarense* (Guan et al. 2018)) or in response to a future climate change conditions such as ocean acidification and solar ultraviolet radiation (*Karenia mikimotoi* (Wang et al. 2019)). In addition, as mentioned above, this animal model had already been used to evaluate *Karlodinium* extract toxicity (employing early-larvae and juveniles) (Deeds et al. 2002, 2006; Peng et al. 2010). In this study, our interest was to evaluate and compare the toxic effect of sublethal concentrations of *K. veneficum* K10 supernatants' extracts containing five novel putative karlotoxins (cand. KmTx-10, cand. KmTx-12, cand. KmTx-13, cand. KmTx-11, and cand. sulfo-KmTx-10; "cand." stands for candidate) (López-Rosales et al. 2015;

Table 1 Bioactivities of the isolated *Karlodinium* toxins

Toxin	Strain	Geographic origin	Hemolytic activity EC_{50}	In vivo effects	In vitro EC_{50} cancer cell	Reference
KmTx-1	CCMP 1974 CCMP 1975	Chesapeake Bay, USA	284 ng mL^{-1} (EF1)	*D. rerio*, *Cyprinodon variegatus* (posthatched larvae); mortality and epithelial damage	2000 ng mL^{-1}	Deeds et al. 2002
KmTx-1	CCMP 1974	Chesapeake Bay, USA	nd	*Danio rerio* (post-hatched larvae), EC_{50} = 800 ng mL^{-1}, epithelial damage	nd	Deeds et al. 2006
KmTx-1	CCMP 2936	Delaware, USA	47 ng mL^{-1} (EH)	nd	nd	Van Wagoner et al. 2008
KmTx-1		Chesapeake Bay, USA	82 ng mL^{-1} (EF1)	nd	nd	Place et al. 2012
KmTx-2	CCMP 2282	South Carolina, USA	368 ng mL^{-1} (EF1)	*Danio rerio* (post-hatched larvae EC_{50} = 800 ng mL^{-1}—juvenile EC_{50} ≈ 400 ng mL^{-1}, gill damage)	nd	Deeds et al. 2006
KmTx-2	KVHU01	Huon River, Australia	343 ng mL^{-1} (EF1)	nd	nd	Mooney et al. 2009
KmTx-2	KVHU01	Huon River, Australia	nd	*Cyprinodon variegatus* (larvae) EC_{50} = 508.2 ng mL^{-1}	nd	Mooney et al. 2010
KmTx-2	CCMP 2064	Georgia, USA	nd	*Danio rerio*, *Cyprinodon variegatus* (juvenile), mortality, and gill damage	nd	Peng et al. 2010
KmTx-2		Chesapeake Bay, USA	1768 ng mL^{-1} (EF1)	nd	nd	Place et al. 2012
KmTx-2	010,410-C6	South Carolina, USA	EF1 (hemolytic activity)	nd	nd	Deeds et al. 2015
KmTx-2	CCMP2778	Longboat Key, Florida USA	1988 ng mL^{-1} (EF2)	nd	nd	Krock et al. 2017
KmTx-2–1	KVSR01	Swan River, Australia	66 ng mL^{-1} (EF1)	nd	nd	Mooney et al. 2009
KmTx-2–2	KVSR01	Swan River, Australia	63 ng mL^{-1} (EF1)	nd	nd	Mooney et al. 2010
KmTx-2–1, KmTx-2–2	AUS#7	Swan River, Australia	EF1 (hemolytic activity)	*Cyprinodon variegatus* (juvenile) mortality	nd	Adolf et al. 2015
KmTx-2–1	KVSR01	Swan River, Australia	nd	*Cyprinodon variegatus* (larvae) EC_{50} = 563.2 ng mL^{-1}	nd	Mooney et al. 2010
KmTx-3	CCMP 2936	Delaware, USA	158 ng mL^{-1} (200 nM) (EH)	nd	nd	Van Wagoner et al. 2010
KmTx-3		Chesapeake Bay, USA	188 ng mL^{-1} (EF1)	nd	nd	Place et al. 2012
KmTx-8	AUS#7	Swan River, Australia	49 nM (EF1)	nd	1064 nM	Waters et al. 2015
KmTx-9	KDCSO15*	Southern Ocean, Australia	3000 nM (EF1)	nd	nd	Waters et al. 2015
65-E-chloro-KmTx-1	CCMP 2936	Delaware, USA	56 nM (EH)	nd	nd	Van Wagoner et al. 2010
10–0-sulfo-KmTx-1	CCMP 2936	Delaware, USA	30 nM (EH)	nd	nd	Van Wagoner et al. 2010
64-E-chloro-KmTx-3	CCMP 2936	Delaware, USA	110 nM (EH)	nd	nd	Van Wagoner et al. 2010

Table 1 (continued)

Toxin	Strain	Geographic origin	Hemolytic activity EC_{50}	In vivo effects	In vitro EC_{50} cancer cell	Reference
10–0-sulfo-KmTx-3	CCMP 2936	Delaware, USA	2400 nM (EH)	nd	nd	Van Wagoner et al. 2010
4,5-dihydro-KmTx-2	GM2	East China Sea	997 ng mL^{-1} (EF2)	nd	15 mM	Cai et al. 2016
4,5-Hihydro-dechloro-KmTx-2	GM2	East China Sea	943 ng mL^{-1} (EF2)	nd	36 mM	Cai et al. 2016
Sulfo KmTx-10	K10, E11	Ebro Delta Bays, Spain	5245 ng mL^{-1} (EF2)	nd	nd	Krock et al. 2017
Karmitoxin	K-0668	Alfacs Bay, Ebro Delta, Spain	nd	*Cyprinodon variegatus* (larvae) mortality	nd	Binzer et al. 2020

EF1 erythrocytes of fish (*Oncorhynchus mykiss*), *EF2* erythrocytes of fish (*Sparus aurata*), *EH* erythrocytes of human, *nd* data not available

* *K. conicum*

Krock et al. 2017) and contribute to the knowledge of the potential effects of this type of ichthyotoxins on the development of fish species in the natural environment.

Materials and methods

Culture of Karlodinium strains

K. veneficum (strain K10) was obtained from the Culture Collection of Harmful Microalgae of IEO, Vigo, Spain. This strain was isolated from an embayment of the Ebro Delta (NW Mediterranean). Inocula were grown at 18 ± 1 °C in a 12:12 h light–dark regime. These cultures were maintained in L1 medium (Guillard and Hargraves 1993) prepared in natural seawater (conductivity of 55 mS; approximately 36.4 ppt) previously sterilized by filtration (0.22 µm). Clonal culture volume was 250 mL. For cultivation, a transparent bubble column photobioreactor (methyl-methacrylate 3.9 mm thick) was used. The column was 8.7 cm in diameter, and the liquid height without gas (H) was fixed at 175 cm. It was inoculated with cells in a stationary phase at an initial concentration of 245,666 cells mL^{-1}. The column was kept illuminated with 58 W fluorescent lamps which allowed for an average irradiance of 200 µmol photon s^{-1} m^{-2} in the bioreactor without any biomass. Photosynthetically active irradiance was measured using a QSL-100 quantum scalar irradiance sensor (Biospherical Instruments, USA). At harvest time (stationary phase), the culture had a concentration of 1,566,000 cells mL^{-1}. An aliquot of 50 mL was preserved in Lugol's iodine solution for determination of cell abundance by microscopic counting. Cell density was determined by settling Lugol's iodine-fixed samples and counting of > 100 cells under an inverted microscope.

Preparation of K. veneficum supernatant extract

A detailed procedure is described elsewhere (Krock et al. 2017). Briefly, the culture´s supernatant was passed through Solid Phase Extraction (SPE) cartridges (LC-18 Supelclean, Sigma-Aldrich, Deisenhofen, Germany). Cartridges were washed with deionized water and 50% aqueous methanol. KmTxs were then eluted with 100% methanol. The methanolic extract was subsequently dried in a gentle N_2 stream and kept at -80 °C until use. From dry and homogeneous samples of *K. veneficum* supernatant's extract (100% MeOH), 10 mg was weighted and then diluted in 500 µL of DMSO. A stock solution of 20,000 mg L^{-1} was obtained. After some preliminary zebrafish bioassays, a supernatant extract concentration range of 0.03 to 6.25 mg L^{-1} (final concentration in assays) was evaluated.

KmTx determination

A dry aliquot of *K. veneficum* supernatants' extract was reconstituted in 500 µL methanol, and the suspension was filtered by centrifugal filtration (Merck Millipore, Ultrafree MC HV, Durapore PVDF 0.45 µm, Eschborn, Germany) for 5 min at 16,100 × g (Centrifuge 5415 R, Eppendorf, Hamburg, Germany). KmTxs were determined by liquid chromatography (LC 1100 chromatograph, Agilent, Waldbronn, Germany) coupled to tandem-mass spectrometry (API 4000 QTrap, Sciex, Darmstadt, Germany) in the selected reaction monitoring (SRM) mode (Krock et al. 2017). All KmTxs were calibrated against an external (noncertified) standard solution of KmTx-2 (23 ng $µL^{-1}$; provided by Allen Place, University of Maryland, USA), and KmTx abundances were expressed as KmTx-2 equivalents.

Hemolysis assay

Hemolysis assays were performed by measuring hemoglobin released from sheep erythrocytes. A protocol optimized for palytoxins (Riobó et al. 2008) was adapted (ouabain was not used as a hemolysis inhibitor). Defibrinated sheep blood was used at a concentration of 45×10^6 erythrocytes per well. Different volumes of the various samples assayed (methanolic extracts) were partially air-dried and then placed in a microtiter's microwell plate to complete the drying. Then, PBS was added (100 µL). After blood was added (100 µL), microwell plates were then incubated for 24 h at 37 °C. PBS and Mediterranean Sea water served as negative controls. Seawater with a salinity of 55 mS was filtered and sterilized (0.2 micron capsule filter was used) (Sartopore® 2 Gamma, Sartorius). In the case of seawater, it followed the same procedure as supernatants (described above). A positive control with distilled water (100% lysis; 100% effect) was also included. An EC_{50} value was obtained. For comparative purposes, saponin was used. All hemolytic assays were carried out in triplicate.

Danio rerio maintenance and egg collection

To obtain eggs for each of the bioassays, zebrafish were reared under standard conditions in the Marine Biotechnology Laboratory of the Faculty of Natural and Oceanographic Sciences (Chile). Photoperiod of 14L:10D and temperature of 27 ± 1 °C was kept. The fish were fed 3 times a day with commercial flake food and once a day with live *Artemia nauplii*. The eggs for the bioassays were placed in plates with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$ prepared in distilled water) and kept at 26.5 °C in a culture chamber.

Evaluation of K. veneficum supernatant's embryotoxicity in zebrafish (Danio rerio)

For these bioassays, 96-well plates with a final volume of 200 µL were used. Bioassays were carried out with the extract obtained from *K. veneficum*'s supernatant with final concentrations of 0.03, 0.3125, and 3.125 mg L^{-1} (total KmTxs expressed as equivalent KmTx-2 were calculated; see Table 3). In addition, a negative control (E3 medium) was run. Assays consisted of three replicates with 5 eggs in the gastrulation phase. The gastrulation phase occurs 5 to 6 h after fertilization depending on the incubation temperature (27–28 °C). Eggs were monitored to quantify different endpoints: (a) percentage of mortality up to 48 h postfertilization when the embryonic development was finished; (b) percentage of hatching success at 76 h postfertilization; and (c) percentage of larval anomalies at 76 h postfertilization. A yolk sac larva is considered alive when heartbeat was observed. This experiment was repeated three times with different egg batches.

Lethal and sublethal effects of K. veneficum's supernatant in zebrafish larvae

Deeds et al. (Deeds et al. 2006) showed that zebrafish larvae exposed to karlotoxins showed damage at the epithelial level. On this background, an experiment was set up to observe possible lethality and sublethal damage in larvae exposed to supernatant extract. Eight days postfertilization larvae (yolk sac depleted) were used. These were exposed for 24 h to *Karlodinium* supernatant extracts and E3 medium (negative control). After the incubation time, the number of alive larvae was quantified, and they were individually mounted on an excavated slide to identify changes in the epithelium (Stereomicroscope Stemi DV4, Zeiss 32X).

Additionally, with the interest of analysing sublethal mechanisms potentially associated with the induction of apoptosis, an in vitro test was implemented. Thus, induction of apoptosis in larvae exposed to different supernatant concentrations was quantified for the sublethality range. Exposure time was 21 h with the positive control (2% v/v ethanol; (Félix et al. 2014) and negative control (E3 medium). For each treatment, a pool of 5 larvae was used. Larvae were disaggregated following the protocol described by Chan and Cheng (2003) with modifications. The suspension of cells was fixed, then centrifuged and resuspended in PBS. Then, cells were assayed with two commercial kits for cytometric analysis: (1) Cell Viability Stain (FDA) and (2) Muse Annexin V & Dead Cell Kit assays (Merck Millipore). The samples were analysed in the Muse Cell Analyzer (Merck Millipore). As suggested by Garcia-Käufer et al., the sizes of the cell populations were standardized in the range of 1.1–1.2 s (Garcia-Käufer et al. 2014). The samples counted on pseudo-replicates for each treatment. This experiment was repeated twice with different larval batches.

Rearing, handling, and experimental work with zebrafish embryos and larvae were carried out under protocols approved by the University of Concepcion's Bioethics Committee and following internationally established procedures.

Statistical analysis

The data analysis was performed with STATISTICA 7. The percentage of mortality and the percentage of live, dead, and apoptotic cells were arcsine transformed. Tests were performed to determine homogeneity variance (Barttlet test) and to assess normality (Shapiro Wilk test). ANOVA was used for parametric data. For the analysis of nonparametric data, the Kruskal–Wallis test followed by multiple comparisons was used.

Results and Discussion

KmTx composition of K. veneficum supernatant

Prior to the toxicity assays, the K10 *K. veneficum*'s supernatant extract was analysed for KmTx composition and revealed a very similar composition with 77.4% KmTx-12 and 14% sulfo-KmTx-10 (Table 2). The KmTx profile of *K. veneficum* strain K10 was first described by Krock et al. recently (Krock et al. 2017). Strain K10 was found to produce four novel, according to HRMS analysis, sulfur containing KmTxs (KmTx-10, KmTx-11, KmTx-12, KmTx-13, and a disulfated form of KmTx-10) with KmTx-12 being the most abundant variant (85% relative abundance) followed by sulfo-KmTx-10 with 10%. All other variants were trace compounds below 5% relative-abundance.

Taking into account that in the cited work (e.g., Krock et al. 2017), KmTx composition in cell extracts was determined, and in this study, KmTxs were determined in the cell-free *K. veneficum*'s supernatant; the similarity of both results indicates that different KmTxs are exuded at the same rate so that intra- and extracellular KmTx compositions are almost identical. Total KmTxs were 25 μg L^{-1} in the supernatant. In addition, in Table 1, the contribution of each cell to the total KmTxs in the supernatant has been calculated, which corresponded to 15.75 fg $cell^{-1}$ of total exudated karlotoxins (in KmTx-2 equivalents) (Table 3).

Culture and hemolytic activity

K. veneficum was cultured in a 10 L bubble column photobioreactor with continuous illumination. Culture conditions are described elsewhere (López-Rosales et al. 2016). To attain a long-stable stationary phase, after 11 days of batch mode growth, concentrated stocks of the medium nutrients were added (fed-batch). The final cell concentration was around 1.5 10^6 cell mL^{-1} (Fig. 1a). In this fed-batch phase, four samples were taken to assess the biomass bioactivity. The saponin equivalent hemolytic effect on erythrocytes (equivalent saponin potency (ESP)) was taken as a proxy of the total content of KmTxs (Krock et al. 2017). As shown in Fig. 1a, ESP kept constant from day 12 to 18 (the pseudo-stationary phase where nutrients were added in fed-batch mode). An amount of 25 μg of KmTx-2 equivalent was obtained per liter of supernatant. In Fig. 1b, the percentage of hemolysis *vs.* concentration of *Karlodinium*'s supernatant extract was plotted. These data allowed us to obtain EC_{50}s values. The calculated cells' EC_{50} was approximately $6{\cdot}10^4$ cells. Thus, each *K. veneficum*'s cell showed an equivalent hemolytic activity of 150 pg saponin. This is much lower (one order of magnitude) than the values obtained previously for the same species (López-Rosales et al. 2016). Different hemolytic activity of the different KmTx congeners is probably responsible for this (see Table 1). For instance, EC_{50} for hemolytic assays conducted with different KmTxs congeners ranged from 47 to 5245 ng mL^{-1} for KmTx-1 and sulfo-KmTx-10, respectively (Table 1). KmTxs in the supernatant studied here showed an EC_{50} of 990 ng mL^{-1}. KmTx-12 of K10 strain used in this work accounted for almost 80% of total KmTxs, similarly to the previous results (Krock et al. 2017). However, López-Rosales et al. found that the same strain the main congeners were KmTx-10 and sulfo-KmTx-10 (López-Rosales et al. 2016). On the other hand, one mL of supernatant showed the same hemolysis as 1.13 μg of saponin. This value is very similar to those previously reported for this species (López-Rosales et al. 2016). In this previous study,

Table 2 Analysis of karlotoxins* in the *K. veneficum* supernatant

	KmTx-10	KmTx-12	KmTx-13	KmTx-11	sulfo-KmTx-10
KmTx in supernatant extract (ng mg^{-1})	8.44	337.62	22.96	5.67	61.69
KmTxs exudated per cell (fg $cell^{-1}$)	0.305	12.19	0.829	0.205	2.23
Percentage (%)	1.93	77.4	5.3	1.3	14.1

Table 3 Assays conducted in wells for supernatant embryotoxicity on *Danio rerio's* evaluation

Supernatant extract's assay concentration assay ($mg{\cdot}L^{-1}$)	Volume of supernatant extracted (mL)	Total KmTx content (pg)	Total KmTx concentration in assay (KmTx $well^{-1}$) expressed as KmTx-2 equivalents	
			($mg{\cdot}L^{-1}$)	($\mu g{\cdot}L^{-1}$)
0.03	0.52	1.29 10^{-2}	$1.29{\cdot}10^{-5}$	0.01
0.3125	5.4	1.35 10^{-1}	$1.35{\cdot}10^{-4}$	0.13
3.125	53.9	1.35	$1.35{\cdot}10^{-3}$	1.35
6.25	107.8	2.69	$2.69{\cdot}10^{-3}$	2.69

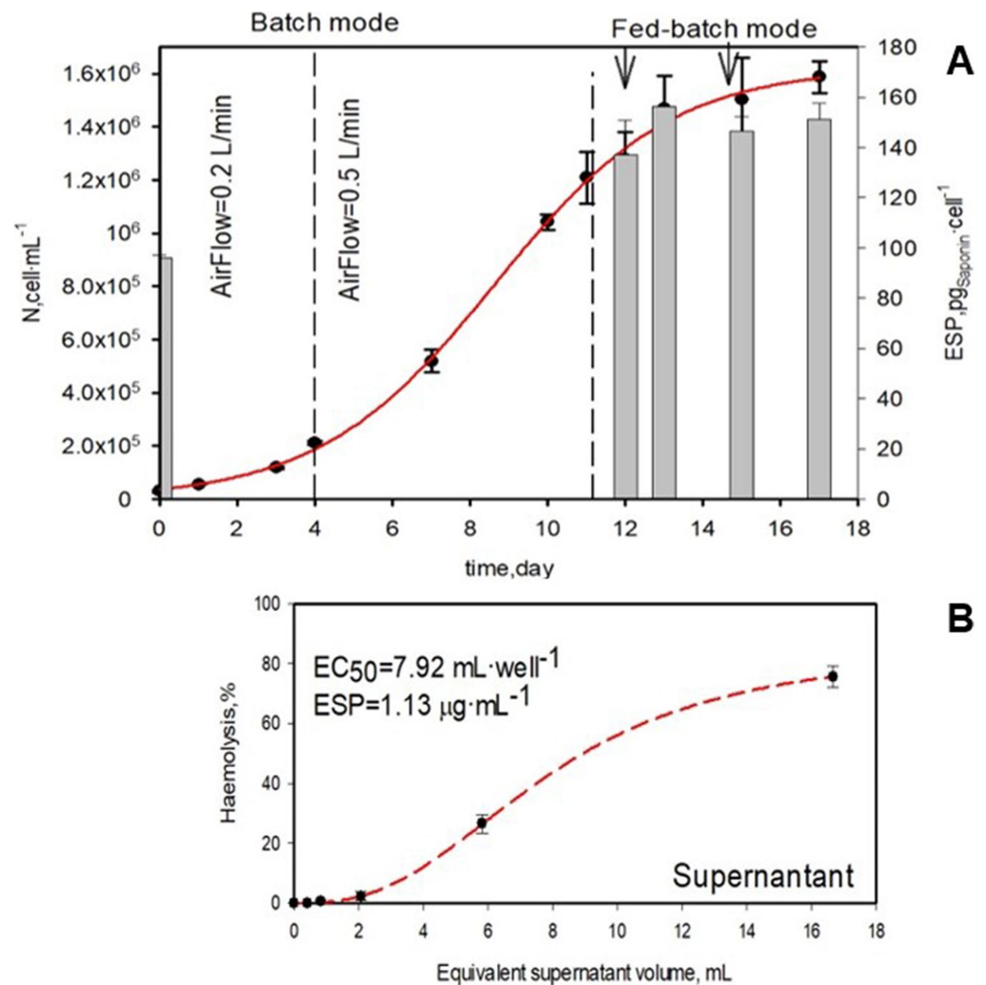

Fig. 1 *Karlodinium veneficum* (K10) culture (**a**) and hemolytic activity of its supernatant (**b**)

toxin analysis was not performed on the supernatant but the hemolytic analysis was performed on different fractions of biomass and collected from a C18 chromatography column. Their results suggested differences in hydrophobicities of the KmTxs from biomass and exudates (López-Rosales et al. 2016). In the present study, different KmTx supernatant concentrations were obtained as was discussed in the previous section showing that KmTx-12 was dominant.

Embryotoxicity of K. veneficum supernatant

In our experiments, it was observed that the supernatant's toxins induced 100% of embryonic mortality at 15 min of exposure at concentrations ≥ 2.69 μg L^{-1} KmTx-2 equivalent (Fig. 2). Detailed observation of the eggs exposed to 2.69 μg L^{-1} KmTx-2 equivalent showed an arrest of the epiboly process after a few minutes. The total disintegration of both the blastoderm and the group of cells that started the migration over the yolk was observed (Fig. 3). Finally, a coagulated egg state was evident (see Fig. 3h).

In the concentration range of 0.01–1.35 μg L^{-1} KmTx-2 equivalent, no embryonic mortality was observed up to 48 h postfertilization; but there was an alteration of the embryo's pigmentation developing. These were almost translucent in comparison with those of the control condition (Fig. 4). During the hatching-starting process with a 3.125 mg L^{-1} (1.35 μg mL^{-1}) treatment, late embryonic mortality was observed thus leading to a lower hatching percentage. This percentage was significantly lower than the one quantified for KmTx-2 equivalent concentrations lower than 1.35 μg mL^{-1} (66.7%; K-W test $p < 0.05$; multiple comparisons $p < 0.05$; Fig. 2). Additionally, in recently hatched larvae, there were anomalies like pericardial oedema and lordosis with different degrees of severity. Both types of larval anomalies occurred in the same high proportion and simultaneously in a high percentage of larvae (87%). Similar teratogenic effects have been described in zebrafish embryos exposed to toxic compounds produced by marine microalgae. For example, in zebrafish embryos exposed to different concentrations of ruptured cells of *Alexandrium tamarense* (Guan et al. 2018) and *Karenia mikimotoi* (Wang et al. 2019), yolk sac oedemas were observed. Thus, marine toxins have been shown to produce embryo coagulation, oedema, and development

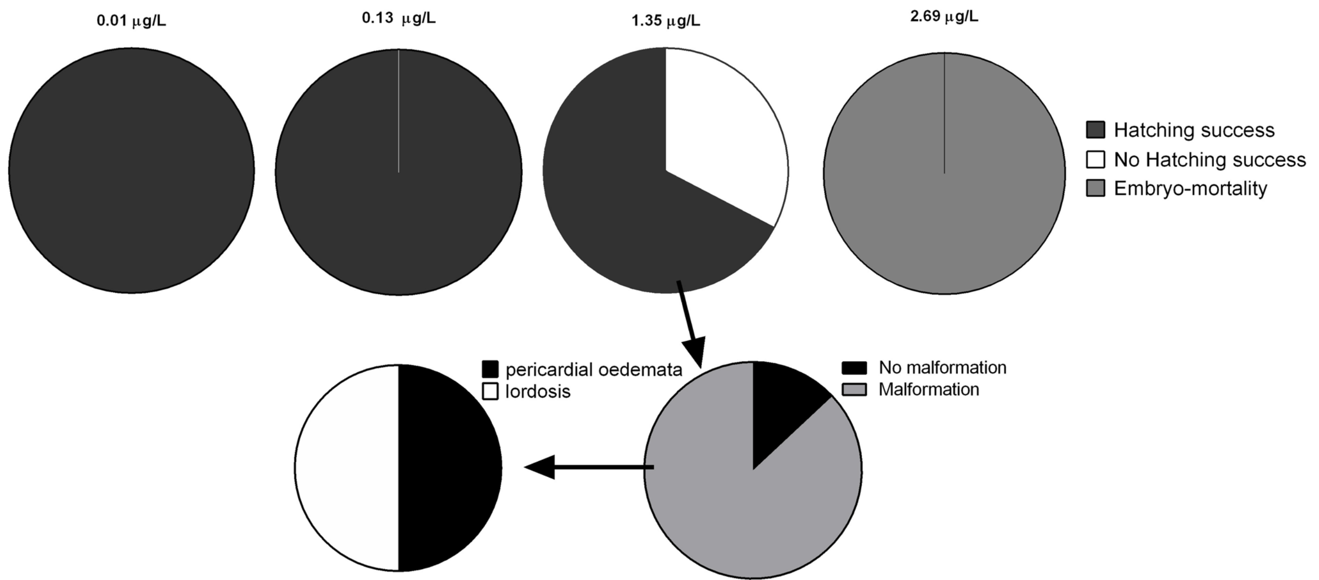

Fig. 2 Effect on mortality, hatching, and presence of anomalies in zebrafish eggs exposed to *K. veneficum* supernatant extract. The amount expressed in µg L^{-1} corresponds to the concentration of the total supernatant extract KmTxs analysed by HPLC–MS and expressed as KmTx-2 equivalent

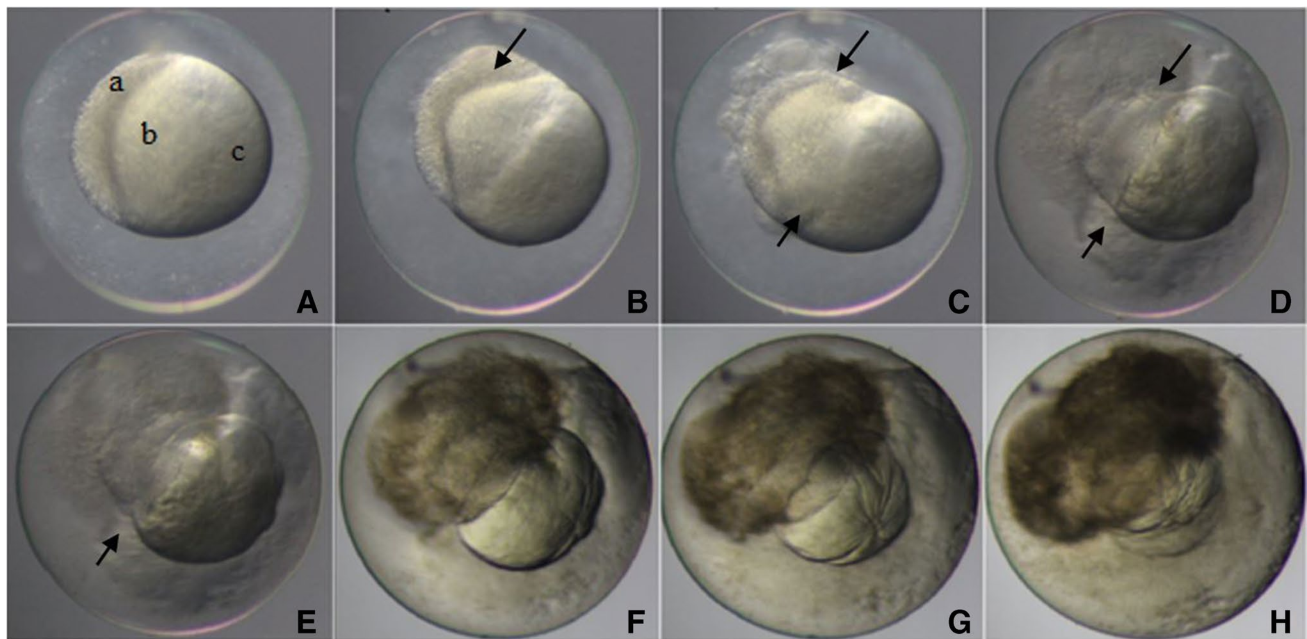

Fig. 3 Zebrafish egg in gastrulation phase exposed to 2.69 µg L^{-1} KmTx-2 equivalent from *K. veneficum* supernatant. Images were captured every 2 min during 15 min of exposure (**a–h**)—**a**: blastoderm, **b**: cell migration, and **c**: yolk. The arrows indicate the disintegration of both the blastoderm and the sheet of cells that should extend over the yolk

retardation and for recently hatched larvae, yolk sac oedema, and bent spine (Guan et al. 2018; Wang et al. 2019). However, Von Hellfeld et al. showed that some of this development anomalies were seen after exposure to a wide variety of chemical compounds (von Hellfeld et al. 2020). For example, the formation of oedema was observed with any of the 45 substances tested in this research. Therefore, oedemata in zebrafish appear to be of very little mechanistic value and should be categorised as an unspecific side effect of both acute and sublethal toxicity.

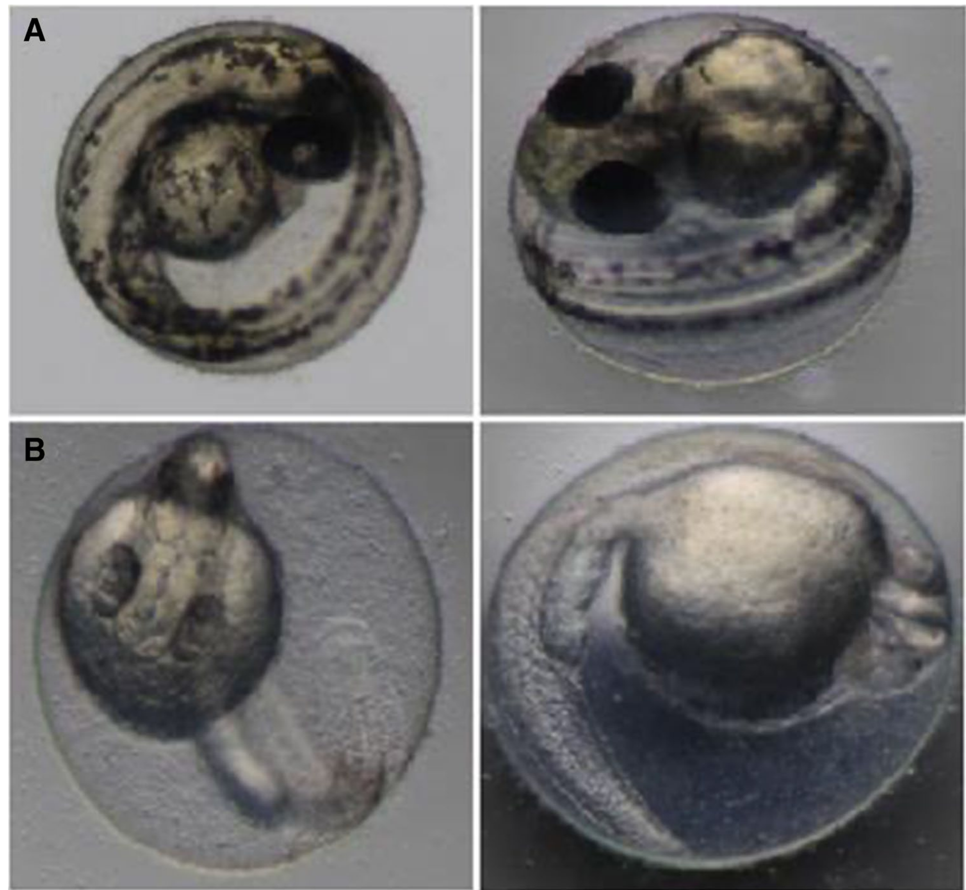

Fig. 4 Advanced stage of zebrafish embryonic development (48 h postfertilization) **a** incubated in control medium (E3 1X) and **b** incubated in 1.35 μg $L.^{-1}$ of KmTxs expressed as KmTx-2 equivalent from *K. veneficum* supernatant (100% MeOH)

Lethal and sublethal effects in zebrafish (Danio rerio) larvae, exposed to K. veneficum supernatant

Concentrations higher than 2.69 μg L^{-1} KmTx-2 equivalent were lethal for early larvae (8 days postfertilization). A 100% larval survival after 24 h of exposure was observed at concentrations lower than or equal to 1.35 μg L^{-1} KmTx-2 equivalent. However, a larvae's decrease in swimming activity was observed 1.35 μg L^{-1} KmTx-2 equivalent, as these were perched at the bottom of the wells. Detailed observation under a microscope showed larval epithelium's damage, being more evident in the lateral-caudal area of the embryonic fin, and its intensity was greater than the one observed in larvae under the positive control condition (2% ethanol; Fig. 5).

Although fourteen karlotoxins congeners have been described until present (see Table 1), ichthyotoxicity has been evaluated considering posthatch larvae (yolk-sac larvae or eleuteroembryo) and juveniles of zebrafish and sheepshead minnow, in just 30% of total congeners. Even though the toxins' effects vary according to life stage and species considered, the available information indicated a higher potency for KmTx-1 and KmTx-2, than KmTx-2–1 or KmTx-2–2 (see Table 1). According to our results, both embryotoxicity and larval mortality indicated that the supernatant of *K. veneficum* strain K10 has a higher potency than that reported for KmTx-1 and KmTx-2 (800 and 400–800 μg L^{-1}, respectively; Table 1).

In the presence of KmTx-1 or KmTx-2, the effect of swelling and sloughing of the epithelium was reported on both fins and gills of the fish exposed (Deeds et al. 2002, 2006; Peng et al. 2010). It can be related to the capacity of karlotoxins to form pores in the cell membranes by binding to membrane lipids. Deeds et al. (2006) reported generalized necrosis of the entire epithelial surface of larval zebrafish induced by both KmTx-1 and KmTx-2 at higher doses (≥ 1 μg L^{-1}). At sublethal concentrations, we expected an increase in nonspecific permeability of multivalent cations such as Ca^{+2}. This could activate apoptosis-specific caspases and provoke alterations in the osmotic balance triggering apoptosis (Deeds et al. 2015; Zhang et al. 2018). To asses this effect, we exposed early larvae (8 days postfertilization) to different KmTx concentrations and evaluated the apoptosis induction using a typical flow cytometry protocol for apoptosis (annexin V-propidium iodide). For all the KmTx-2 equivalent concentrations (0.01 μg L^{-1}) assayed, apoptotic cells were observed. However, after comparing the percentages of cells undergoing apoptosis (early + late), no statistically significant differences were found between

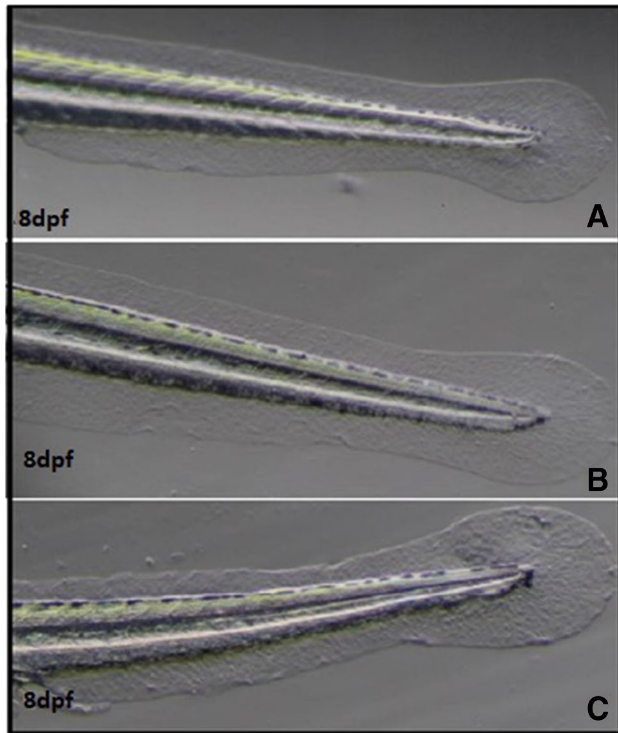

Fig. 5 Detail of epithelial tissue on the caudal region of zebrafish larvae (8 days postfertilization). **a** Negative control (Medium control, E3 1X). **b** Positive control (2% ETOH). **c** 1.35 µg $L.^{-1}$ of KmTxs from *K. veneficum* supernatant (100% MeOH)

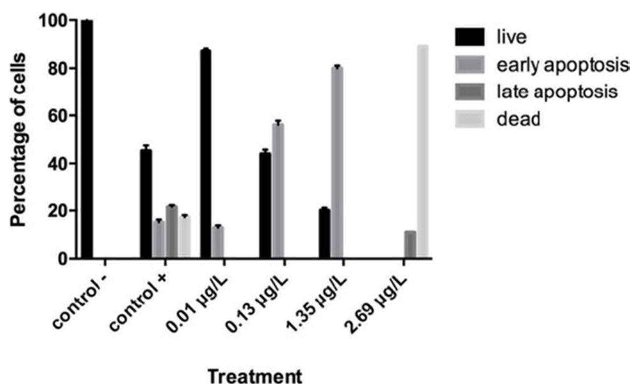

Fig. 6 Percentage of live, death, and apoptotic cells from disaggregated zebrafish larvae prior exposed to increasing concentrations of KmTxs from *K. veneficum*'s supernatant

treatments (Fig. 6, ANOVA, $p = 0.685$). On the other hand, it was observed that increasing concentrations of KmTxs resulted in a decrease of live cells' percentage and a proportional increase of cells' percentage in the early-apoptosis stage, reaching 80% in larvae exposed to 1.35 µg L^{-1} of KmTx-2 (equivalent). In larvae exposed to ethanol (positive control for induction of apoptosis), 39% of the cells were in late apoptosis or dead; percentage only observed for larvae exposed to 2.69 µg L^{-1} KmTx-2 (equivalent) at the end of the experiment. Finally, it is suggested that the karlotoxin (KmTx) mode of action is dependent on the sterol composition of the target cell membranes (Pradhan and Ki 2022). Possibly, the ability of KmTx to cause the formation of pores in cell membranes would trigger apoptosis at sublethal concentrations (below EC 50) and necrosis at greater concentrations (above EC 50) (Deeds et al. 2006, 2015).

Deeds et al. (2006) observed severe degeneration of gill tissue including oedema, enlargement, and necrosis, with curling or loss of secondary lamellae in a detailed description of histopathological effects in fish exposed to the KmTx-2. These effects could be related to the capacity of KmTx-2 to induce cell lysis through colloid osmolysis, presumably through membrane permeability changes (Deeds et al. 2015). Our results showed that karlotoxins produced by *Karlodinium veneficum* (strain K10) induced apoptosis in the range of sublethal concentrations. Apoptosis has been described as a toxic effect triggered by microalgae toxins such as okadaic acid (Dietrich et al. 2020), azaspiracids (Ferreiro et al. 2017), or cyanotoxins (e.g., microcystin (Zeng et al. 2014)). Recent studies on cyanotoxins showed that microcystin-LR induced apoptosis by activation of endoplasmic reticulum stress (Qi et al. 2016). In addition, an adverse effect on zebrafish embryos' development in presence of nodularin was evidenced, which may be associated with oxidative stress and apoptosis through the activation of the P53-AX/BCL-2-CASPASE 3-mediated pathway (Chen et al. 2020).

If it is considered that in a *Karlodinium veneficum* bloom, cell densities can reach 10^4–10^5 cells mL^{-1} (Place et al. 2012) and that each cell produces 15.75 fg of toxins (KmTx-2 equivalents) that are exuded to the medium; in the water column, there could be between 0.16 and 1.6 µg L^{-1} KmTx-2 equivalents. Similar concentrations were shown for seawater samples from *K. micrum*-associated fish kills (Deeds et al. 2006). Our results indicate that in this range of concentrations both lethal (embryonic mortality) and sublethal effects (embryonic malformations, epithelial damage, and induction of apoptosis) are evidenced in the early stages of the species model *Danio rerio*. For apoptosis induction, concentrations as low as 0.01 µg L^{-1} were likely sufficient to trigger these processes in fish.

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Author contribution All authors contributed to the study conception and design, commented on versions of the manuscript, and read and approved the final manuscript. Material preparation, data collection, and analysis were performed by Katia Álvarez-Muñoz, Lorenzo López-Rosales, and Juan José Gallardo-Rodríguez. The first draft of the manuscript was written by Alejandra Llanos-Rivera, Juan José Gallardo-Rodríguez, Allisson Astuya-Villalón, Bernd Krock, and Katia Álvarez-Muñoz; Francisco García-Camacho and Asterio Sánchez-Mirón revised and edited the final draft.

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Data availability Data are available within the article or its supplementary materials.

Declarations

Ethics approval Rearing, handling, and experimental work with zebrafish embryos and larvae were carried out under protocols approved by the University of Concepcion's Bioethics Committee and following internationally established procedures.

Consent to participate Not applicable.

Consent to publish All authors consented to publish the results.

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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