J Parasit Dis (Jan-Mar 2019) 43(1):46–53
https://doi.org/10.1007/s12639-018-1056-1

ORIGINAL ARTICLE

Toxicity of formalin for fingerlings of *Cyprinus carpio* var. *koi* and in vitro efficacy against *Dactylogyrus minutus* Kulwièc, 1927 (Monogenea: Dactylogyridae)

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Received: 24 August 2018 / Accepted: 12 November 2018 / Published online: 14 December 2018

Abstract The toxicity of formalin on *Cyprinus carpio* var. *koi* and its anti-parasite effects against *Dactylogyrus minutus* (Monogenea) in in vitro tests is analyzed. Specimens of *D. minutus* were submitted to eight concentrations of formalin: 50, 75, 100, 125, 150, 175, 200, 250 mg L^{-1}, in triplicate. Concentrations of formalin 100, 150 and 200 mg L^{-1} were then tested to determine the median lethal concentration of 50% of the fish per immersion bath. Fish behavior was also observed during the first 6 h of exposure. The 200 mg L^{-1} concentration was the most rapid efficacy for *D. minutus*, killing all parasites in 16 min. All parasites were killed in 47 min at concentration 100 mg L^{-1}. Concentration 200 mg L^{-1} was the most lethal for fish in less than 24 h exposure, with 24 h LC_{50} at 135.44 (119.78–153.14) mg L^{-1}. The therapeutic index was 2.05–30 min and 1.15–16 min. A short bath (1 h) is recommended in koi carp with a minimum concentration of 75 mg L^{-1} of formalin, not exceeding 100 mg L^{-1} for treatment against *D. minutus.*

Keywords *Cyprinus carpio* · Treatment · Formalin · Therapeutic index · Monogenea · *Dactylogyrus* sp.

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Introduction

The koi carp *Cyprinus carpio* (Linnaeus, 1758) is an ornamental fish with high market demand due to its ease in breeding and to its great variation in color patterns (Hussain et al. 2014, 2015). It is a fast-growing species (Hashem et al. 1997) and very tolerant to variations in water quality parameters and stocking density (Carneiro et al. 2015). However, its cultivation has been affected by ectoparasite Monogenea *Dactylogyrus* Diesing 1850, featuring 900 species (Gibson et al. 1996; Santos et al. 2016).

Carps are usually infected by more than one species of *Dactylogyrus*, some of them highly pathogenic, especially for fingerlings (Kritsky and Heckmann 2002; Jalali and Barzegar 2005), causing massive mortality rates and high liabilities to producers (Buchmann et al. 1993; Bretzinger et al. 1999; Kritsky and Heckmann 2002). Several studies investigated the biology and mechanisms of infestation of these parasites (Ergens and Dulmaa 1969; Dzika et al. 2009; Mhaisen et al. 2013), pinpointing the species *D. achmerowi* Gussev 1955, *D. anchoratus* Dujardin, 1845, *D. arcuatus* Yamguti, 1942, *D. difformis* Wegener, 1857, *D. extensus* Mueller & Van Cleave, 1932, *D. formosus* Kulwiec, 1927, *D. intermedius* Wegener, 1910, *D. vastator* Nybelin, 1924 and *D. minutus* Kulwiec, 1927 as the most prevalent (Gibson et al. 1996; Jarkovský et al. 2004; Kohn et al. 2006; Stojanovski et al. 2008; Molnár 2012).

Outbreaks of Monogenea parasites occur due to their life cycle in a specific host, temperature and culture densities (Turgut and Akin 2003). Monogenea are included among parasites with higher dispersion, epizootic impact and control difficulties in tanks (Pereira et al. 2006). Koi carp fingerlings were devastated in Israel by a severe infestation of *Dactylogyrus vastator* Nybelin, 1924, during the spring and early summer (Paperna 1963).

For the control of Monogenea outbreaks, several chemical agents, such as praziquantel, toltrazuril, chloramine, hydrogen peroxide, malachite green, formalin and others, have been used to verify their efficacy in in vivo treatments,. However, few studies were undertaken to investigate in vitro effects, or rather, the products' performance when added directly to adult parasites or at some stage of their life cycle (Schmahl and Mehlhorn 1985; Buchmann et al. 1993; Buchmann and Kristensson 2003; Stephens et al. 2003; Sharp et al. 2004; Katharios et al. 2006; Sitjà-Bobadilla et al. 2006).

Formalin (aqueous formaldehyde gas 37%), which replaced malachite green in the treatment of parasitic diseases, has been highlighted for its effectiveness (Martins 2004; Buchmann and Kristensson 2003). It also provides other advantages, such as easy metabolization by aquatic organisms and low potential for bioaccumulation (Picón-Camacho et al. 2012). Formalin´s main drawback is its high toxicity to fish. However, recovery time is relatively short when properly applied, even in small fish (Schmahl 1991). The use of formalin has recently been banned in the European Union because the handling of the product causes risks to humans (UEMS 2016).

Formalin is used as a fungicide for fish eggs and as a treatment against ectoparasite infestations. It has been legally registered since 1986 in the United States and Canada (Rach et al. 1997; Jenkins et al. 2014). Formalin is particularly applied against protozoa (*Ichthyobodo* sp., *Trichodina* sp. and *Chilodonella* sp.) and Monogenea (Dactylogyridae and Gyrodactylidae) parasites of the body surface and of fish gills (Scott 1993).

The use of formalin is indicated in some protocols in the form of short baths (up to 1 h) at concentrations ranging between 150 and 250 mL m^{-3} or in long-term baths (24 h) at concentrations ranging between 10 and 15 mL m^{-3} (Thoney and Hargis 1991; Martins 2004). However, there is scant information on the direct effect of this product on the parasite and on the concentrations that are really effective and safe for fish. Fish resistance to chemicals may vary and depends on a number of factors, including species, age and culture conditions (Dolezelova et al. 2009).

Thus, treatment with formalin has been investigated for use in commercial fish farms, analyzing its efficacy, toxicity and which doses are safe for the fish species. In other words, better prophylaxis and treatment protocols and procedures are investigated (Burka et al. 1997).

Current study evaluates the Median lethal LC_{50} concentration of formalin in *Cyprinus carpio* var. *koi* and its in vitro anti-parasitic activity against *Dactylogyrus minutus* Kulwièc, 1927.

Materials and methods

Specimen collection

One hundred and fifty koi carp fingerlings (weight 1.73 ± 0.25 g; total length 4.7 ± 0.46 cm mean ± standard deviation) were obtained in September 2016 from a fish farm located in Biguaçu, state of Santa Catarina, Brazil (27°27′20″S; 48°41′32″W), and were transported alive to the Aquatic Organisms Health Laboratory of the Federal University of Santa Catarina (UFSC). The fish were distributed in 70L-aquariums and acclimatized under experimental conditions (dissolved oxygen 6.95 ± 0.63 mg L^{-1}; water temperature 27.82 ± 0.14 °C; conductivity 213 ± 46.66 µS cm^{-1}, total suspended solids 106 ± 23.33 ppm and total ammonia 0.60 ± 0.63 mg L^{-1}). All parameters, except total ammonia, were measured with a multiparameter Hanna® HI9829 (Hanna Instruments Brazil, Barueri, Brazil). Total ammonia was measured with a colorimetric kit (Alfakit, Florianópolis, Brazil).

After the acclimatization period, thirty fish were anesthetized with eugenol (75 mg L^{-1}) and euthanized by cerebral disruption to investigate Monogenea infestation in the gills, with a stereomicroscope (ZEISS Stemi DV4). All procedures were performed following protocols by the Ethics Committee of the Federal University of Santa Catarina (CEUA/UFSC/PP00928).

Morphological identification of *Dactylogyrus minutus*

Prior to the in vitro assays, dactylogyrids were collected from the gills of 30 fish; they were fixed in 70% alcohol, washed in distilled water in a Petri dish and mounted in Hoyer's medium, following procedures by Kritsky et al. (1995) for the study of sclerotized structures. Some dactylogyrids were stained with Gomori trichrome stain (Humason 1979) to study their internal morphology. Criteria followed the morphological characteristics by Ogawa and Egusa (1977) and Lambert (1977). Ecological terms "prevalence" (P%), "mean intensity of infection" (IMI) and "average abundance" (AM) followed Bush et al. (1997).

In vitro formalin test against *Dactylogyrus minutus*

Eight formalin concentrations were tested to detect the most efficient minimal concentration causing 100% parasite mortality according to time intervals. Stock solutions of formalin diluted in distilled water were previously prepared and tested separately in triplicate: 50, 75, 100, 125,

150, 175, 200, 250 mg L^{-1} (37% Formaldehyde, SIGMA-ALDRICH®). In addition, two controls were prepared, or rather, the system water in which the fish were (C1) and the other was made up of distilled water (C2).

Filaments from the parasitized gill arches and containing specimens of *D. minutus* (5–6 parasites per well) were collected and separated into a flat bottom plate from six-well cell culture (KASVI®). In the standardized volume of 3 mL per well, each parasitized filament received a formalin concentration (adapted from Hashimoto et al. 2016). Whereas initial time (0 min) was the moment of immersion of the gill filaments in each concentration tested, finish time occurred when total mortality occurred. Mortality was observed every 5 min at the highest concentrations and every 10 min at the lowest concentrations, by stereomicroscope (ZEISS Stemi DV4, Oberkochen, Germany). Parasites were considered dead when the absence of movements was detected when stimulated with a fine histological needle, and by the observation of typical characteristics of Monogenea mortality, such as body wrinkling (Reimschuessel et al. 2011). After analyzed the results, the parasites were mounted in Hoyer's medium for confirmation of the species *D. minutus*. Number of dead Monogenea parasites was also employed to determine the median effective concentration (EC_{50}). EC refers to the drug concentration which produces 50% of its maximum response (Rose and Golan 2015).

Toxicity of formalin for koi carp

Seventy-two specimens were randomly distributed in polyethylene tanks with a fixed volume of 25 L, totaling eight fish per tank to evaluate the effect of formalin on koi carp. Each tank contained a thermostat at an average temperature of 26 °C and air-to-air compressor adjusted to the maximum of its capacity. Formalin baths were subsequently submitted to three different concentrations of 100, 150 and 200 mg L^{-1} (37% Formaldehyde, SIGMA-ALDRICH®) in triplicate. Formaldehyde was added to each tank at the specific amount to obtain formalin concentrations described above and mixed with a glass stick for 60 s. Feeding of the fish was suspended 24 h before the start of the tests and during the experimental period. Dead fish were removed and quantified up to 24 h or when mortality reached 50%. Median lethal LC_{50} concentration was calculated for 6 and 24 h. Survival fish at the end of the 24 h period remained under observation within the same system until a total of 168 h. The therapeutic index (TI) was calculated using the ratio fish LC_{50}/EC_{50} of the parasite: $TI = \frac{LC50}{EC_{50}}$ (Foster 1939). So that a drug may be considered good for therapeutic purposes, it should have TI equal to or higher than 2 (Yacubian 2007). The observation of fish behavior in the first 6 h was also recorded. The fish were observed by swimming rhythm and agitation on the surface or near the aeration site.

Statistical analysis

Data from the in vitro test of the concentrations with respect to time were submitted to Shapiro–Wilk and Bartlett to verify the normality and homoscedasticity, respectively. Data that not presented variance homogeneity were transformed in Log. After the premise was ensured, the data were submitted to a variance analysis unifactorial ANOVA and the means were separated by a Tukey test. All of the tests were realized at a significance value of 5% with the of Statistica 10.0 software. Parasite EC_{50} and koi carp LC_{50} were determined by the *Trimmed–Spearman–Karber* method for recording mean mortality rates (Hamilton et al. 1977).

Results

Morphological identification of *Dactylogyrus minutus*

Dactylogyrus minutus (Fig. 1) presented the same characteristics of the species described by Ogawa and Egusa (1977) with a prevalence of 80%; mean infection intensity of 4.26 ± 2.55; average abundance 5.33 ± 1.52.

In vitro formalin test against *Dactylogyrus minutus*

Among all concentrations, the 200 mg L^{-1} concentration was the most rapid efficacy, killing all parasites in 16 min. However, there was no statistical difference for 250, 175, 125 and 75 mg L^{-1} when compared to the most lethal concentration (Table 1). At concentration 100 mg L^{-1}, parasites died in 47 min, below what is usually recommended in prophylactic baths (1 h for short-term baths). The two controls took more than 5 h to kill the parasites. The lowest concentration tested (50 mg L^{-1}) required 104 min to kill all *D. minutus* specimens. Formalin EC_{50} for *D. minutus* at 16 min was 114.04 (95.68–135.92) mg L^{-1} and decreased to 66.02 (52.81–82.52) mg L^{-1} at 30 min (Fig. 2).

Toxicity of formalin for koi carp

The concentration of 200 mg L^{-1} was the most lethal for all fish in less than 24 h. Koi carp showed did not die during exposure to 100 mg L^{-1} formalin concentration (Fig. 3). However, almost all fish (70%) were dead at 150 mg L^{-1}, in 24 h. LC_{50} for 6 h was 191.34 (174.75–209.51) mg L^{-1} and LC_{50} 24 h was 135.44 (119.78–153.14) mg L^{-1} (Table 2).

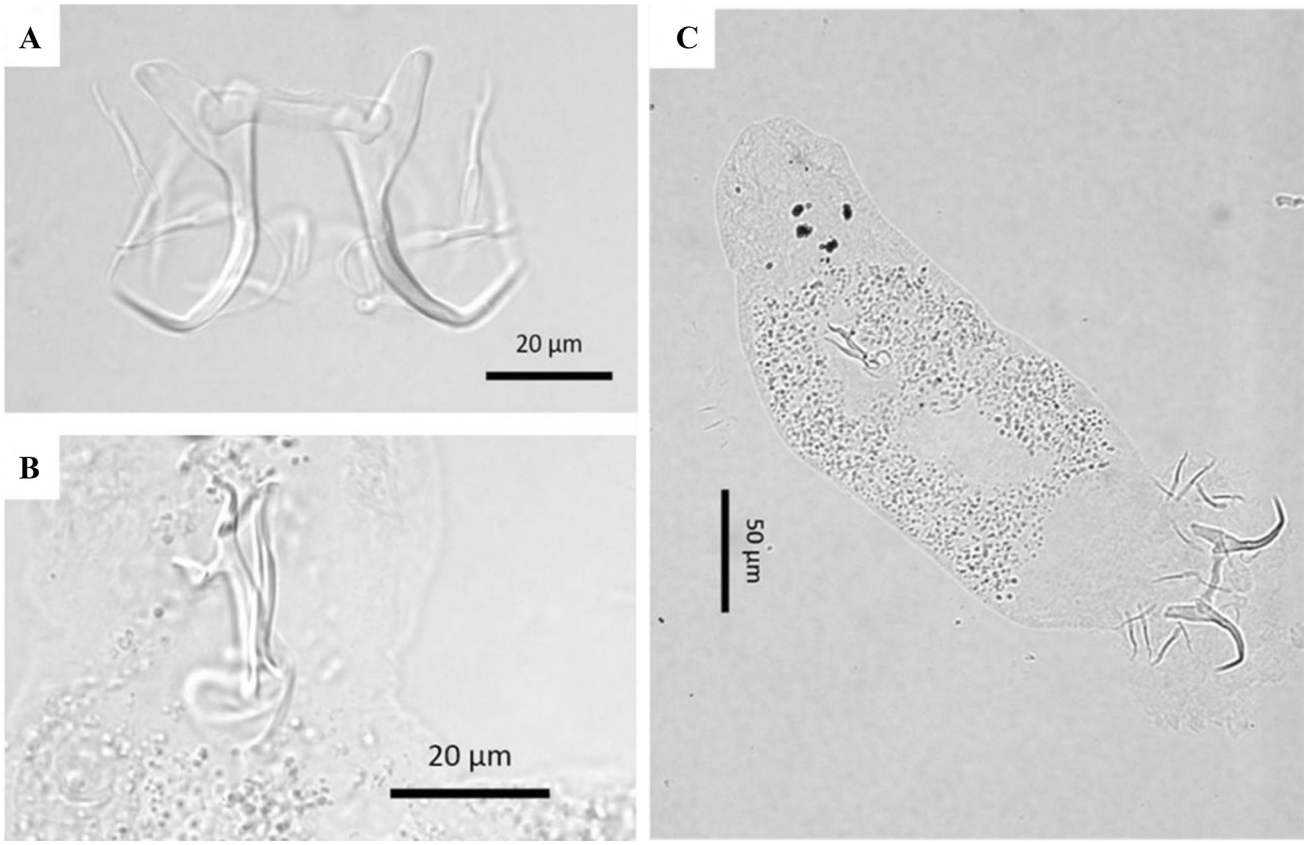

Fig. 1 Light photomicrograph of *Dactylogyrus minutus* Kulwièc, 1927, of *Cyprinus carpio* var. *koi* collected in Biguaçu, Santa Catarina, Brazil. **a** Haptor; **b** male copulatory complex; **c** adult parasite

Table 1 Concentration of formalin in mg L^{-1} capable of killing 100% of *Dactylogyrus minutus* (Monogenea: Dactylogyridae) considering the time, in the in vitro test

Formalin (mg L^{-1})	Time (min)
C1	318 ± 12.19^{e}
C2	269 ± 15.10^{d}
50	104 ± 6.18^{c}
75	37 ± 9.29^{ab}
100	47 ± 3.46^{b}
125	38 ± 2.00^{ab}
150	20 ± 1.00^{a}
175	29 ± 4.50^{ab}
200	16 ± 3.60^{a}
250	20 ± 450^{ab}

C1: system water; C2: distilled water

Different letters indicate a significant difference among the times ($p < 0.05$)

Taking into account the value of LC_{50} 24 h of koi carp and EC_{50} for *D. minutus*, the result of the therapeutic index was between 1.15 for 16 min and 2.05 for 30 min.

Fish behavior during baths was gradually observed in the first 6 h, at all concentrations. During the first 2 h, the behavior of the fish at the 200 mg L^{-1} concentration comprised agglomeration close to the aeration entrance in the boxes and agitated swimming with hyperventilation. Increase in mucus production was observed after more than 4 h of exposure, causing a change in the color of the water (slightly cloudy); decrease in opercular beating and slow swimming until the onset of the first deaths. In addition, the discoloration of fish, especially the most colored ones, was well evidenced. At 150 and 100 mg L^{-1}, the first signs of agitation and agglomeration near the inflow in the boxes started between 4 and 6 h after the start of the baths. No mortality was recorded in these two concentrations until the initial 6 h of behavioral observation.

Discussion

The results elucidate the potential of formalin in in vitro treatment against *D. minutus*, parasite of *C. carpio* var. *koi*. Several previous studies available in the literature were developed to establish therapeutic concentrations of the chemical agent that were safe for the host. They were

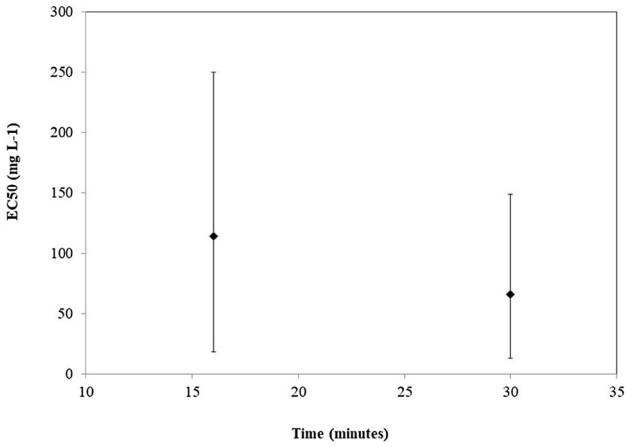

Fig. 2 Effective median concentration (EC_{50}) of formalin for *Dactylogyrus minutus* (Monogenea: Dactylogyridae) infesting *Cyprinus carpio* var. *koi* (Bars represent 95% of the confidence limit)

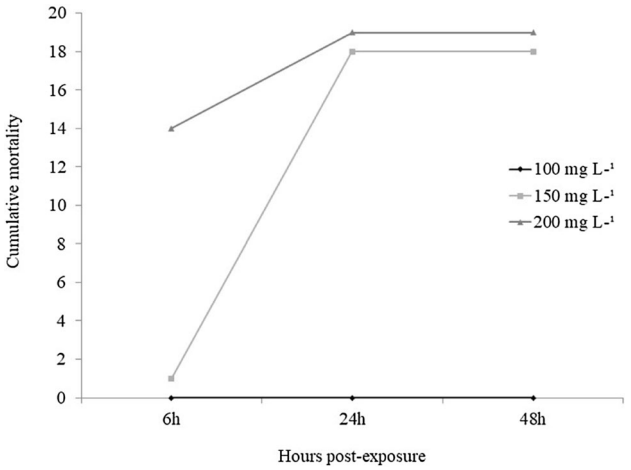

Fig. 3 Cumulative mortality of *Cyprinus carpio* var. *koi* relative to the time during formalin immersion baths at different concentrations

Table 2 Median lethal concentration (LC_{50}) of formalin (mg L^{-1}) for *Cyprinus carpio* var. *koi* during the immersion baths for 6 and 24 h

	6 h	24 h
LC_{50} mg L^{-1}	191.34	135.44
95% upper limit	174.75	119.78
95% lower limit	209.51	153.14

tested directly in the form of immersion baths (Martins 2004). However, when tested as above, concentrations could only be partially effective (efficacy ranging between 49 and 78%). Moreover, direct effective concentration on the parasite is unknown (Liang and Leong 1992; Sharp et al. 2004; Fajer-Ávila et al. 2007).

The most lethal concentration in current study in relation to time intervals was 200 mg L^{-1} for parasites and fish. Complete elimination of *D. minutus* occurred in 16 min. The above concentration may be satisfactory in the case of *C. carpio* larvae. In fact, results by Theron et al. (1991) showed that larvae of the common carp at 4, 12 and 20 days after hatching, proved to be very resistant in formalin baths at 200 mg L^{-1}, for 30 min. Tamaru et al. (2001) suggest formalin treatment for swordtail (*Xiphophorus helleri* Heckel, 1848) at concentration 250 mg L^{-1}, for 1 h. Further, 250 mg L^{-1} formalin in the gills of the rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) presented aggressive effects on fish after 1 h bath, such as detachment of secondary lamella epithelium, hyperplasia and cell hypertrophy (Smith and Piper 1972). Therefore, regardless of the concentration, preliminary tests on a small batch of fish are important due to possible variations in product toxicity on a particular species, which may increase by water quality, size or age of the specimens.

In this study, the most lethal concentrations killed the parasites in less than 30 min, but these doses may damage the integrity of fish tissue during the prophylactic bath. Fajer-Ávila et al. (2003) observed that, in lethal dose trials with bullseye puffer (*Sphoeroides annulatus* Jenyns, 1842), fish showed hyperemia in the mouth and tail erosion at concentrations above 142 mg L^{-1} after 20 min of bath. According to Reardon and Harrell (1990), formalin toxicity causes dysfunction in the osmoregulatory and respiratory balance, leading the fish to death. Further, Chinabut et al. (1988) observed fish swimming on the surface with erratic movements and reported first mortalities, after 12–18 h of formalin treatment.

Rowland et al. (2006) reported that several fish submitted to 30 or 40 mg L^{-1} formalin presented partial reinfestation after 30 days of treatment. In 2 months, the prevalence of Monogenea returned to 100%. Diggles et al. (1993) performed tests on the life cycle phase of Monogenea and reported a decrease in the viability of laying eggs, the survival of oncomiracidia and a 70% reduction in *Polylabroides multispinosus* adults and juveniles from the yellowfin bream gills (*Acanthopagrus australis* Günther, 1859), submitted to 300 mg L^{-1} formalin during 30 min. Nevertheless, the authors still recommend a larger dose (400 mg L^{-1}) to ensure complete removal of adult parasites. Although formalin is effective against Monogenea eggs, its greatest efficacy appears to be in newly placed eggs than in embryonated eggs about to hatch (Svendsen and Haug 1991).

Monogenea parasites of freshwater or saltwater fish may react at different concentrations of formalin. Pahor-Filho et al. (2012) observed that, in the case of mullet juveniles (*Mugil liza* Valenciennes, 1836), 400 mg L^{-1} formalin were lethal to *Ligophorus* spp. after a 1-h bath. In the case of the silver perch (*Bidyanus bidyanus* Mitchell, 1838), a

30 mg L^{-1} concentration of formalin was the most effective against *Lepidotrema bidyana* (Rowland et al. 2006). Sharp et al. (2004) evaluated 250 and 400 mg L^{-1} of formalin against *Benedenia seriolae* and *Zeuxapta seriolae* from the gills of the yellowtail amberjack (*Seriola lalandi* Valenciennes, 1833) and detected better results (90%) at the highest concentration, with a 1-h bath.

In the control test with fish tank water and distilled water, *D. minutus* remained alive for more than 5 h outside the host. Similar results obtained by Andrade-Porto et al. (2017) revealed approximately the same lifetime for *Dawestrema cycloancistrium* Price and Nowling, 1967, or rather, mortalities began after 4 h from the start of the tests. Several studies report that temperature is one of the factors that most influence Monogenea survival and reproduction success.

According to studies by the Food and Drug Administration (FDA) (1995), the maximum recommended formalin concentration for a prophylactic bath in fish of any species is 250 mg L^{-1} for 1 h exposure, at a temperature below 27 °C and dissolved oxygen above 4 mg/L. Since current results indicate that 200 mg L^{-1} was lethal in less than 24 h for koi carp, the recovery time of the fish after treatment, besides the damage caused in the gill epithelium, may be irreparable, even considering a 1 h bath. At concentration 200 mg L^{-1}, the fish showed signs of agglomeration, accelerated opercular beating, lethargy and increased mucus production in less than 6 h exposure. This explains why, after tests at the lower concentrations, some specimens survived and were able to recover when allocated to another system.

One may conclude that, in formalin treatments, fish susceptibility depends on concentration, time, species and age. Rates for the control of the same parasite group may vary between wide concentration ranges (Sharp et al. 2004).

FDA (1995) report shows that the formalin time interval in water is approximately 36 h. After that period, it falls drastically due to the effects of hydrolysis and oxidation. Time interval may reach 48 h under anaerobic conditions (Alberta Environment 2006). After the employment of formalin, the water of any concentration treatment should be diluted to 1 mg L^{-1} before being discharged into the environment or into another water body (FDA 1995).

The therapeutic index (TI) (Foster, 1939) is an approximate safe measure for a chemical to be used for treatment purposes, calculated by the LC_{50} ratio of the fish and EC_{50} of the parasite. In the case of koi carp, 135.44 mg L^{-1} of formalin is a safe dose and, at the same time, effective against the parasite as from 30 min of exposure, according to the recorded TI of 2.05. An anti-parasite drug must have a high therapeutic index to be effective at all stages of the life cycle, not to promote resistance of the parasite and to be of low environmental impact (Shao 2001). The higher the index, the safer the dose, since it prolongs exposure time to the chemical by employing a fixed concentration without affecting the health of the fish (Horwath et al. 1978).

Overall results show that a minimum concentration of formalin against *D. minutus* is 75 mg L^{-1} and the koi carp was well resistant at 100 mg L^{-1} formalin concentration in a short bath (1 h). Other points, such as the monitoring of water quality during bathing, are required, since formalin decreases the dissolved oxygen level in water (Reed et al. 2009).

Acknowledgements The authors thank National Council of Scientific and Technological Development (CNPq) for financial support (CNPq 446072/2014-1) and Research grant to M.L. Martins (CNPq 305869/2014-0), Coordination for the Improvement of Higher Education Personnel (CAPES/EMBRAPA Ed. 15/236) for PhD scholarship to K.R. Tancredo, and Vale dos Betas fish farm for fish donation. This study as financed in part by the Coordination for the Improvement of Higher Education Personnel—Brazil (CAPES)—Finance Code 001.

Authors' contributions KRT: written the article, in vitro and in vivo test. NCM: in vitro and in vivo test. SP: statistical analysis. MLM (advisor): critical review of the article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were performed following protocols by the Ethics Committee of the Federal University of Santa Catarina (CEUA/UFSC/PP00928).

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