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In vitro efficacy of emamectin benzoate and glyphosate against monogenean parasites from *Colossoma macropomum*

[*Eficácia* in vitro do *benzoato de emamectina e do glifosato contra parasitos monogenéticos de* Colossoma macropomum]

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ABSTRACT

This study investigated the *in vitro* efficacy of emamectin benzoate and glyphosate against monogeneans from *Colossoma macropomum* and tolerance of this species to these therapeutic drugs. *In vitro* assays demonstrated that concentrations of emamectin benzoate (12.5, 16.6, 20.8 and 25.0g/L) and glyphosate (2,000; 3,000; 4,000 and 5,000mg/L) were 100% effective against *Anacanthorus spathulatus*, *Notozothecium janauachensis* and *Mymarothecium boegeri*. The highest concentrations of emamectin benzoate (20.8 and 25.0g/L) were 100% effective after 30 minutes of exposure. For glyphosate, the highest concentrations were the most effective, and 4,000 and 5,000 mg/L immobilized 100% of parasites in 45 minutes and 30 minutes, respectively. Scanning electron microscopy revealed that the parasites exposed to 20.8 and 25.0g/L of emamectin benzoate and 5,000 mg/L of glyphosate presented integument covered with deeper wrinkles. Behavioral changes occurred in *C. macropomum* exposed to all concentrations of both drugs, were as mortality occurred following exposure to emamectin benzoate and glyphosate at 2.0,12,5 and 25.0g/L and 500, 1,000, 2,000, 3,000, 4,000 and 5,000mg/L, respectively. Low concentrations of emamectin benzoate and of glyphosate were tolerated by *C. macropomum*, but these concentrations may have not efficacy against infection of monogeneans.

Keywords: anthelminthic, ectoparasites, infection, tambaqui, tolerance.

RESUMO

Este estudo investigou a eficácia in vitro *do benzoato de emamectina e do glifosato contra monogenéticos de* Colossoma macropomum *e a tolerância dessa espécie a essas drogas terapêuticas. Ensaios* in vitro *demonstraram que as concentrações de benzoato de emamectina (12,5; 16,6; 20,8 e 25,0g/L) e de glifosato (2.000; 3.000; 4.000 e 5.000mg/L) foram 100% eficazes contra* Anacanthorus spathulatus, Notozothecium janauachensis *e* Mymarothecium boegeri. *As maiores concentrações de benzoato de emamectina (20,8 e 25,0g/L) foram 100% efetivas após 30 minutos de exposição. Para o glifosato, as maiores concentrações foram as mais efetivas, sendo que 4.000 e 5.000 mg/L imobilizaram 100% dos parasitos em 45 minutos e 30 minutos, respectivamente. A microscopia eletrônica de varredura revelou que os parasitos expostos a 20,8 e 25,0g/L de benzoato de emamectina e 5.000 mg/L de glifosato apresentaram tegumento coberto por rugas mais profundas. Alterações comportamentais ocorreram em* C. macropomum *exposto a todas as concentrações de ambas as drogas, como mortalidade após exposição a benzoato de emamectina e glifosato a 2,0;12,5 e 25,0g/L e 500; 1.000; 2.000; 3.000; 4.000 e 5.000mg/L, respectivamente. Baixas concentrações de benzoato de emamectina e de glifosato foram toleradas por* C. macropomum, *mas essas baixas concentrações podem não ter eficácia contra infecções por monogenéticos.*

Palavras-chave: anti-helmíntico, ectoparasitas, infecção, tambaqui, tolerância

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INTRODUCTION

Monogeneans are ectoparasites with a direct, typically short life cycle. Adult parasites continuously lay eggs that hatch into infectious, free-living larvae (oncomiracidia) that reach juvenile and adult stages once they settle onto host fish. These characteristics of monogeneans can lead to high infection levels in farmed fish (Morales-Serna *et al*., 2018; Alves *et al*., 2019). These ectoparasites have caused significant economic losses for marine and freshwater fish production in different countries (Tavares-Dias and Martins, 2017; Huston *et al*., 2020). Monogeneans are therefore a threat for the cultivation of many freshwater fish, including tambaqui *Colossoma macropomum* (Morales-Serna *et al*., 2018; Alves *et al*., 2019), an Amazonian fish of great importance for fish farming (Braz-Mota *et al*., 2015). Hence, the use of control strategies based on accepted principles and applicable strategies in fish farming are essential to avoid diseases caused by monogeneans.

The management of infections caused by monogeneans in fish farms is challenging because of the current limited availability of efficacious licensed products, which can be exacerbated by the development of resistance to chemical antiparasitic drugs in some monogenean populations (Tojo *et al*., 1992; Zhu, 2020). Different antiparasitic drugs have been used to keep farmed fish free from infections by monogeneans and therefore to support intensive fish farms, but there is wide range efficacy and tolerance in fishes (Tojo *et al.*, 1992; Morales-Serna *et al*., 2018; Alves *et al*., 2019; Merella *et al*., 2021; Tavares-Dias, 2021a,b). Hence, it is essential to look for alternatives to current chemical treatments because of the reduced efficacy of these treatments over time. Glyphosate and emamectin benzoate yet are not used as anthelmintic drugs for the control of infections caused by monogeneans and could represent novel treatment options against these parasites in farmed fish.

Glyphosate (N-(phosphonomethyl) glycine) is an herbicide used in agriculture farms to eliminate all types of plants, including weeds, grasses, and shrubs. This herbicide has been tested to control infective cercariae stages of trematodes in snails *Potamopyrgus antipodarum* (Kelly *et al*., 2010; Hock and Poulin, 2012). Monte *et al*. (2016) demonstrated that exposure to glyphosate affected the life cycle of the trematode *Echinostoma paraensei*, causing mortality of miracidia and cercariae and reducing development of eggs. However, this chemical drug has not yet been used for controlling monogenean infection in fish.

Emamectin benzoate is an avermectin that acts as an acaricide, bactericide and insecticide used to control different pests in crops (Kumar *et al.*, 2022). This avermectin has been approved by the United States Food and Drug Administration and European Medicines Agency for use in aquaculture (Raja *et al*., 2022). Additionally, emamectin benzoate has been widely used in control and treatment against crustacean parasites species in farmed fish, administered orally in the feed (Gustafson *et al*., 2006; Saksida *et al*., 2010; Jones *et al*., 2012; Raja *et al*., 2022). Emamectin benzoate has been also used, *in vivo* trial, with efficacy in against endohelminths of fish such as nematodes *Pseudocapillaria tomentosa* (Collymore *et al*., 2014) and *Philometra rubra* (Béland *et al*., 2020), as well as *in vitro* trial against acanthocephalans *Neoechinorhynchus buttnerae* (Oliveira *et al*., 2019). However, the effects of both drugs in monogeneans of fish have not tested. In addition, since fish are sensitive to the presence of drugs that are used as chemotherapeutants, the tolerance of fish should also be tested. Thus, this study was conducted to evaluate the *in vitro* efficacy of emamectin benzoate and glyphosate against monogeneans, and the tolerance of these drugs for *C. macropomum*.

MATERIAL AND METHODS

Colossoma macropomum fingerlings (n = 250) were obtained from a commercial fish farm in Macapá, in the state of Amapá (Brazil), and maintained in the Embrapa Amapá Aquaculture and Fishery Laboratory (Brazil). The fish were kept in a 500 L tank with constant aeration and continuous water renewal (1.1 L/min) and were fed twice daily with a commercial feed containing 32% crude protein (Guabi®, Brazil). The following water parameters were monitored every two days: mean temperature (29.1 ± 0.1°C), dissolved oxygen (5.6 ± 0.2 mg/L), pH (5.4 ± 0.2), total ammonia (0.5 ± 0.2 mg/L), alkalinity (10.2 ± 0.001 mg/L) and hardness

(10.0 ± 0 mg/L). The tank was siphoned weekly to remove accumulated organic matter, and the water was renewed.

These fish, which were naturally infected by monogeneans, were used in all assays.

This study was developed in accordance with the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and with authorization from the Ethics Committee in the Use of Animals of Embrapa Amapá (Protocol N^{o} 013-CEUA/CPAFAP).

A commercial formulation of Roundup® Original DI (Monsanto company, São Paulo, Brazil) containing 815g/L of glyphosate [N-(phosphonomethyl) glycine] as the active ingredient. Emamectin benzoate of Proclaim®-50 (Syngenta, São Paulo, Brazil) containing 50 g/kg of emamectin benzoate as the active ingredient.

Ten *C. macropomum* fingerlings with mean weight of 79.3±31.4g and mean length of 16.6±1.9 cm were euthanized by medullary section and their parasitized gills removed and used to determine what duration of exposure to the four different concentrations of emamectin benzoate and glyphosate. Gill arches of naturally infected fish by monogeneans were removed and placed individually in Petri dishes. One gill arch was immersed in each concentration of emamectin benzoate (12.5, 16.6, 20.8 and 25.0g/L) (Oliveira *et al*., 2019) or glyphosate (2,000; 3,000; 4,000 and 5,000mg/L), with three replicates for each treatment (3 gill arches by treatment). One control group with cultivation tank water and three replicates for each treatment (3 gill arches by treatment) was used.

Under a stereomicroscope, fields of view containing ± 20 monogeneans were selected in each Petri dish, and after submerging the gill arches in the different concentrations of anthelminthic drugs, the parasites were observed at 5 minutes intervals to count the number of live and dead monogeneans. The parasites were considered dead when they were detached from the gill tissue or when they were attached to the gill tissue but had completely lost their mobility and in absence of movement when stimulated with a needle (Soares *et al*., 2016). The efficacy of each treatment was estimated as proposed by Zhang *et al*. (2014). We recorded the time it took to kill 100% of the monogeneans and hypothesized that a treatment was effective if 100% parasite mortality was achieved. The monogeneans were collected and prepared for identification according to methods of Eiras *et al.* (2006).

Based on *in vitro* results, different concentrations were used to evaluate the tolerance of *C. macropomum* to both anthelminthic drugs.

At the end of the *in vitro* assays, the gill arches used in the treatments with emamectin benzoate (12.5, 16.6, 20.8 and 25.0g/L) and glyphosate (2,000; 3,000; 4,000 and 5,000mg/L), and control group were fixed in 2.5% glutaraldehyde prepared in a phosphate buffer of 0.1 M pH 7.2. Then, the gills were subjected to three washes in 0.1 M phosphate buffer at 15 min intervals. The samples were dehydrated in increasing concentrations of ethyl alcohol (70, 80, 90, 96, 100 and 100), for 10 minutes at each concentration (Luz *et al*. 2021). The samples were analyzed and photomicrographed using a Scanning Electron Microscope (Hitach, Tokyo, Japan, Mod. TM3030Plud) in the Pharmaceutical Research Laboratory from Federal University of Amapá (UNIFAP).

Fish tolerance was tested based on *in vitro* results to determine the optimal concentration for therapeutic baths with emamectin benzoate and glyphosate. The tolerance assays were carried out with 180 C. *macropomum* fingerlings (122.7 ± 32.2 g and 18.8 ± 1.6 cm). Each treatment consisted of three replicates with five fish per replicate (15 fish per treatment) using 100/L tanks. Treatments were carried out with different concentrations of the emamectin benzoate (0.1, 0.5, 2.0, 12.5 and 25.0 g/L) and of glyphosate (250; 500; 1,000; 2,000; 3,000; 4,000 and 5,000 mg/L). The tanks of 80 L used for the tolerance assays were maintained with no water renewal and only with constant aeration.

Changes in fish behavior (i.e., opercular movement, caudal beat, and response to mechanical stimuli) and mortality were observed with four hours of exposure to the different concentrations of emamectin benzoate and glyphosate.

RESULTS

The gills of *C. macropomum*, used in *in vitro* assays, were parasitized by monogeneans *Anacanthorus spathulatus* Kritsky, Thatcher & Kayton, 1979, *Mymarothecium boegeri* Kritsky Thatcher & Kayton, 1979 and *Notozothecium janauachensis* Belmont-Jégu, Domingues & Martins, 2004. Monogeneans exposed to cultivation tank water (Figure 1A) presented a defined body shape with shallow wrinkles on the surface. However, parasites exposed to the 20.8 and 25.0 g/L of emamectin benzoate showed tegument covered with deeper wrinkles (Figure 1B-C), while parasites exposed to 5,000 mg/L of glyphosate showed damages (perforation) in the tegument of monogenean (Figure 1D).

At concentrations of 12.5 g/L of emamectin benzoate, the immobilization of 100% of the parasites occurred after 1 h of exposure and with 16.6 g/L immobilization occurred at 45 min. At concentrations of 20.8 and 25.0 g/L of emamectin benzoate, the immobilization of 100% of the parasites occurred after 30 min (Table 1 and Figure 2). At concentrations of 2,000 and 3,000 mg/L of glyphosate, the immobilization of 100% of the parasites occurred after 45 and 60 min, respectively. At concentrations of 4,000 and 5,000 mg/L of glyphosate, the immobilization of 100% of the parasites occurred after 45 min and 30 min, respectively (Table 1 and Figure 3). In the control group using only cultivation tank water, all parasites were dead after 9 h (Table 1).

Table 1. *In vitro* antiparasitic action of emamectin benzoate and glyphosate against monogeneans in relation to the concentration and time of exposure

Time of exposure	Treatments	Live parasites	Mortality (%)
0 h	Control with water of tank	27.7 ± 6.1	0
3 h	Control with water of tank	25.3 ± 8.2	8.4
5 h	Control with water of tank	23.0 ± 10.2	16.9
8 h	Control with water of tank	6.7 ± 1.9	80.7
9 h	Control with water of tank	0	100
0 h	12.5 g/L of emamectin benzoate	26.0 ± 7.1	0
5 min	12.5 g/L of emamectin benzoate	23.3 ± 8.5	10.3
15 min	12.5 g/L of emamectin benzoate	16.0 ± 10.7	38.5
30 min	12.5 g/L of emamectin benzoate	2.0 ± 1.4	92.3
45 min	12.5 g/L of emamectin benzoate	0.3 ± 0.5	98.7
60 min	12.5 g/L of emamectin benzoate	0	100
0 h	16.6 g/L of emamectin benzoate	23.0 ± 2.2	0
5 min	16.6 g/L of emamectin benzoate	22.3 ± 2.6	2.9
15 min	16.6 g/L of emamectin benzoate	13.7 ± 2.6	40.6
30 min	16.6 g/L of emamectin benzoate	2.3 ± 2.1	89.9
45 min	16.6 g/L of emamectin benzoate	0	100
0 h	20.8 g/L of emamectin benzoate	20.7 ± 0.9	0
5 min	20.8 g/L of emamectin benzoate	15.0 ± 4.1	27.4
15 min	20.8 g/L of emamectin benzoate	3.7 ± 2.6	82.3
30 min	20.8 g/L of emamectin benzoate	0	100
5 min	25.0 g/L of emamectin benzoate	18.3 ± 6.2	21.4
15 min	25.0 g/L of emamectin benzoate	1.0 ± 1.4	95.7
30 min	25.0 g/L of emamectin benzoate	0	100
5 min	2,000 mg/L of glyphosate	18.7 ± 1.7	8.2
15 min	2,000 mg/L of glyphosate	10.3 ± 6.1	49.2
30 min	2,000 mg/L of glyphosate	2.0 ± 2.8	86.9
45 min	2,000 mg/L of glyphosate	0	100
0 h	3,000 mg/L of glyphosate	20.7 ± 0.9	0
5 min	3,000 mg/L of glyphosate	20.7 ± 0.9	0
15 min	3,000 mg/L of glyphosate	18.7 ± 1.2	9.7
30 min	3,000 mg/L of glyphosate	12.3 ± 4.1	40.3
45 min	3,000 mg/L of glyphosate	4.0 ± 4.3	80.6
60 min	3,000 mg/L of glyphosate	0	100
0 h	4,000 mg/L of glyphosate	20.3 ± 1.2	0
5 min	4,000 mg/L of glyphosate	17.7 ± 4.2	13.1
15 min	4,000 mg/L of glyphosate	6.0 ± 3.3	70.5
30 min	4,000 mg/L of glyphosate	1.0 ± 1.4	95.1
45 min	4,000 mg/L of glyphosate	0	100
0 h	5,000 mg/L of glyphosate	21.0 ± 1.4	0
5 min	5,000 mg/L of glyphosate	13.7 ± 3.4	34.9
15 min	5,000 mg/L of glyphosate	0.7 ± 0.9	85.7
30 min	5,000 mg/L of glyphosate	0	100

During the tolerance assays with glyphosate and emamectin benzoate, the fish showed behavioral changes at all concentrations, as shown in Table 2. In addition, in fish exposed to glyphosate and emamectin benzoate, loss of mucus and irritation in the nostrils were observed.

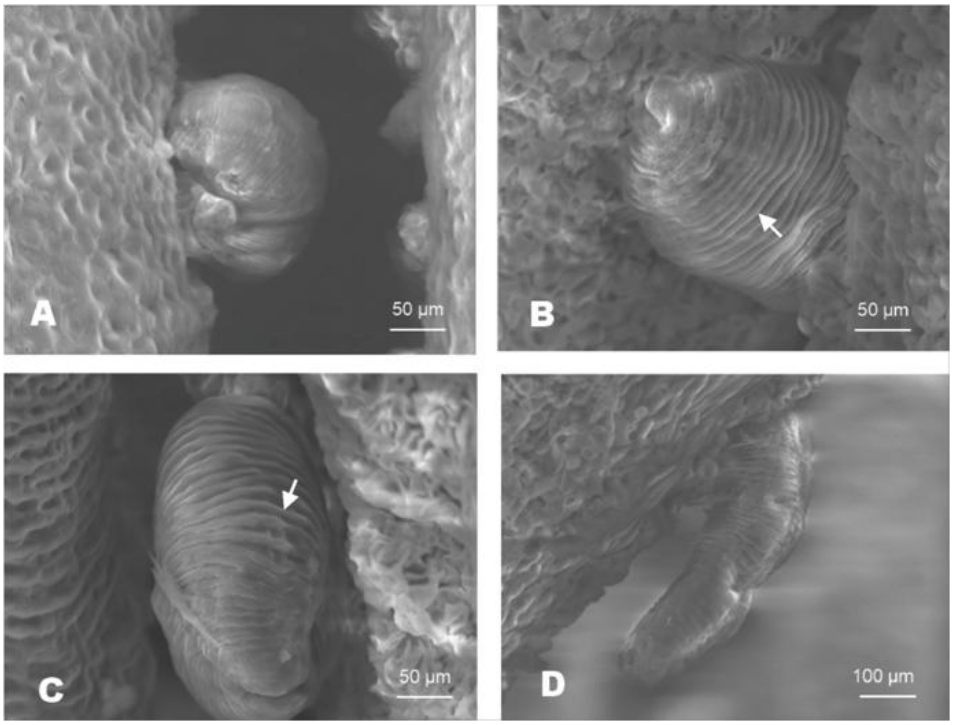

Figure 1. Scanning electronic microscopy for monogeneans of *Colossoma macropomum* exposed to emamectin benzoate and glyphosate. (A) Parasites after 9 hours of exposure to cultivation tank water. (B) Parasite after 60 min of exposure to 25.0 g/L of emamectin benzoate showing the wrinkles on the parasite integument (arrow). (C)Parasite after 45 min of exposure to 20.8 g/L of emamectin benzoate showing the wrinkles on the parasite integument (arrow). (D) Parasite after 60 min of exposure to 5,000 mg/L of glyphosate.

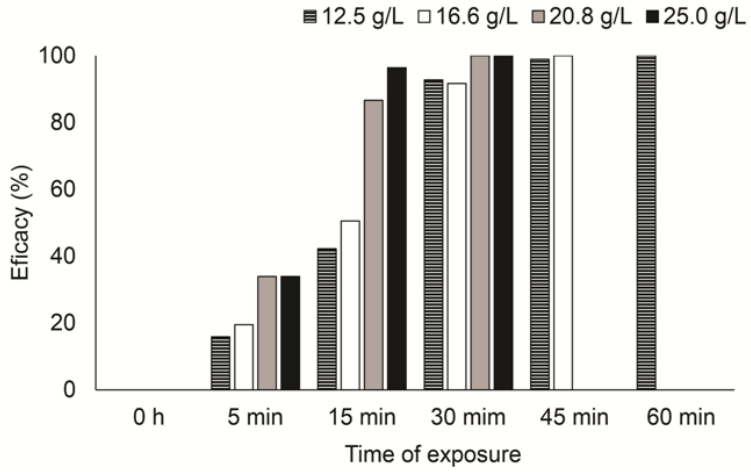

Figure 2. *In vitro* efficacy of different concentrations of emamectin benzoate against monogeneans of *Colossoma macropomum.*

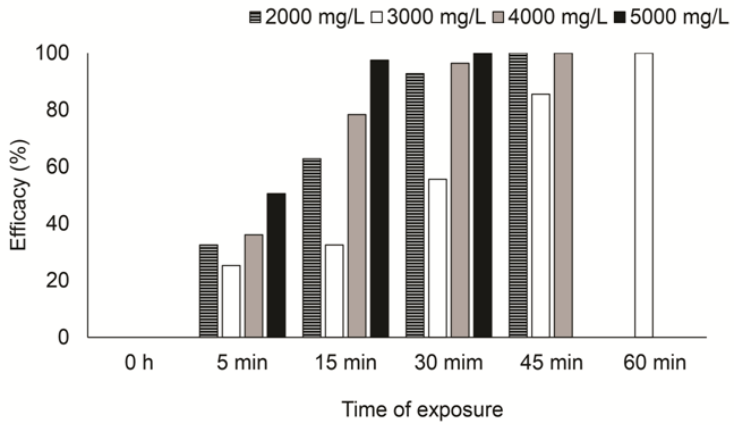

Figure 3. *In vitro* efficacy of different concentrations of glyphosate against monogeneans of *Colossoma macropomum.*

Table 2. Tolerance and behavior alterations of *Colossoma macropomum* exposed to different concentrations of emamectin benzoate and the glyphosate

Concentration (g/L)	Mortality (%)	Exposure time	Behavioral changes of fish
0.1 g/L of emamectin benzoate	0	4 h	Agitation and accelerated opercular beat
0.5 g/L of emamectin benzoate	0	4 h	Agitation, accelerated opercular beat, shallow breathing and loss of equilibrium
2.0 g/L of emamectin benzoate	100	55 min	Agitation, accelerated opercular beat, shallow breathing, lethargy, loss of equilibrium and death
12.5 g/L of emamectin benzoate	100	23 min	Agitation, accelerated opercular beat and swimming, shallow breathing, lethargy, loss of equilibrium and death
25.0 g/L of emamectin benzoate	100	20 min	Agitation, jumps, accelerated opercular beat and swimming, shallow breathing, lethargy, loss of equilibrium and death
250 mg/L of glyphosate	0	4 h	Reduction in opercular beat and shallow breathing
500 mg/L of glyphosate	100	4 h	Agitation, accelerated opercular beat and swimming, shallow breathing, lethargy, loss of equilibrium and death
1,000 mg/L of glyphosate	100	52 min	Agitation, jumps, accelerated opercular beat and swimming, shallow breathing, lethargy, tipping over and death
2,000 mg/L of glyphosate	100	35 min	Agitation, jumps, accelerated opercular beat and swimming, shallow breathing, lethargy, loss of equilibrium and death
3,000 mg/L of glyphosate	100	23 min	Agitation, jumps, accelerated opercular beat and swimming, shallow breathing, lethargy, loss of equilibrium and death
4,000 mg/L of glyphosate	100	20 min	Agitation, jumps, accelerated opercular beat and swimming, shallow breathing, lethargy, loss of equilibrium and death
5,000 mg/L of glyphosate	100	18 min	Agitation, jumps, accelerated opercular beat and swimming, shallow breathing, lethargy, loss of equilibrium and death

DISCUSSION

Reduced sensitivity and potential resistance to some available chemotherapeutic drugs are constant threats for maintaining the control of monogeneans in fish farms all around the world. The monitoring of the efficacy of treatments with chemotherapeutics, together with reasons for any apparent reduction in their efficacy performance, are essential. Most chemotherapeutic treatments show decreasing efficacy over time, together with the use of increasing concentrations, because of the development of resistance of monogenean populations. Therefore, strategic rotation of chemotherapeutants should be encouraged (Tojo *et al*., 1992) and empirical evidence for the benefit of such strategies more fully evaluated. Prospective efficacy studies of novel chemotherapeutant drugs should be based on several treatment episodes to register in regulation organs of chemotherapeutant for aquaculture. Furthermore, they are normally strictly controlled and often should have balanced data sets. Hence, these laboratory-based studies are essential in establishing the *in vitro* efficacy of glyphosate and emamectin benzoate, due to current interest in developing culture of *C. macropromum* is important to implementing strategies to control monogeneans. The *in vitro* assays showed that all concentrations of emamectin benzoate (12.5, 16.6, 20.8 and 25.0 g/L) and of glyphosate (2,000; 3,000; 4,000 and 5,000 mg/L) tested had anthelmintic activity against *A. spathulatus*, *M. boegeri* and *N. janauachensis*, since 100% of the monogeneans were death. According to Collymore *et al.* (2014), the action of avermectins on helminths is paralysis of parasites, as verified in the present study with emamectin benzoate. However, the action of glyphosate on parasites needs to be studied.

After *in vitro* exposure of monogeneans to emamectin benzoate or glyphosate, deeper wrinkling, damage, and perforation in the tegument were observed. Similar results were reported for monogeneans exposed to *Copaifera reticulata* oleoresin (Malheiros *et al*., 2020), and to the essential oil of *Alpinia zerumbet* (Luz *et al*., 2021), *Piper callosum*, *Piper hispidum* and *Piper marginatum* (Alves *et al*., 2021). The tegument of monogeneans plays an essential role in their survival, such as absorption and secretion of substances, osmoregulation, and mechanical support (Dalton *et al*., 2004; Luz *et al*., 2021). Thus, may be detrimental to the monogeneans following exposure to emamectin benzoate and glyphosate.

As the tolerance of chemotherapeutants can vary depending on the fish species and the treatment conditions (Tavares-Dias, 2021a,b), the tolerance of glyphosate and emamectin benzoate was determined for C. *macropomum* before the application of therapeutic baths for the *in vivo* control of monogeneans. This is particularly important as the recommended concentrations and treatment times for some chemotherapeutants are near the lethal level for certain farmed fish species. The higher concentrations of glyphosate (500-5,000 mg/L) used were not tolerated by juvenile *C. macropomum* (122.7 g), differing from results where concentrations of 0.1-0.5 g/L of emamectin benzoate and 250 mg/L of glyphosate were used. The high concentrations of emamectin benzoate (2.0, 12.5 and 25.0 g/L) and of glyphosate (500; 1,000; 2,000; 3,000; 4,000 and 5,000 mg/L) were highly toxicant, causing mortality and changes in behavior in fish exposed. For *Oreochromis niloticus*, seven consecutive days of feeding with 50 and 500 µg/kg fish/day of emamectin benzoate caused 2.2 and 4.4 % mortality, and fish exposed to this higher concentration exhibited abnormal swimming behavior and darkened body color (Julinta *et al*., 2020). For *Labeo rohita*, exposure to 500-1,000 µg/L of emamectin benzoate caused slow and erratic movement, lethargy, surfacing, loss of balance, body flattened, exhibited respiratory distress and mortality of fish (Kumar *et al*., 2022). For this same Indian fish species exposed to 0.5-0.8 mg/L of glyphosate, Ghaffar *et al.* (2021) reported clinical signs and behavior changes but no mortality. Such concentrations of glyphosate were similar to those tolerated by *C. macropomum* of this study. Therefore, these behavioral changes may be considered sensitive indicators of exposure to both chemical drugs.

Another concern with the use of chemotherapeutic agents in fish farming has been human exposure with the consumption of fish meat. A maximum residue limit of 100 µg/kg of emamectin benzoate in the edible tissue of fish has been established by the European Union (Julinta *et al*., 2020). Turnipseed *et al.* (2018) demonstrated that the emamectin benzoate takes

about three days to depurate in fish muscle. In fish, according to the FDA/EPA, the maximum residue limit of glyphosate is 0.25 mg/L (Ujowundu *et al*., 2017). Although the glyphosate has been classified as a low toxicity by the Brazilian Health Surveillance Agency (ANVISA) and non-carcinogenic to humans according to the European Food Safety Authority (Santos *et al*., 2017), care must still be used when handling this product commonly used in agriculture. In addition, it has been discussed that the bioconcentration factors for glyphosate to be very low because of its high solubility and photodegradation in water and low octane-water partition coefficient, making glyphosate readily excreted by the fishes (Ujowundu *et al*., 2017).

In conclusion, as high concentrations of emamectin benzoate (2.0-25.0 g/L) and of glyphosate (500-5,000 mg/L) were toxic to fish, these concentrations should not be used. Concentration of 0.1-0.5 g/L of emamectin benzoate and 250 mg/L of glyphosate were tolerated by *C. macropomum*, but these low concentrations may have not efficacy in the control of infections by monogeneans.

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