Ciência Animal, v.34, n.1, p.88-98, jan./mar., 2024.

SODIUM CHLORIDE AND FORMALIN TO CONTROL MONOGENOIDS OF COLOSSOMA MACROPOMUM IN THE PERUVIAN AMAZONIA

(*Cloreto de sódio e formalina no controle de monogenóides de Colossoma macropomum na Amazônia peruana)*

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ABSTRACT

Colossoma macropomum is one of the main fish species farmed in the Peruvian Amazonia, being highly demanded and accepted by the local population. Among the pathogens that parasitize freshwater fish, species of Monogenoidea stand out. Therefore, the following study aimed to evaluate the efficacy of sodium chloride (salt) and formalin as a treatment against monogenoids present in C. macropomum cultivated in the Peruvian Amazonia. The species of Monogenoidea identified in this study were Anacanthorus spatulathus, A. penilabiatus, and Notozothecium janauachaensis. In vitro and in vivo tests were carried out in the "Laboratorio de Parasitología y Sanidad Acuícola" located in Iquitos, Peru. Regarding the in vitro assay, 20 and 30 mg ml $^{-1}$ of salt and 0.005 and 0.008 mg ml $^{-1}$ of formalin were tested. The mean survival time of the monogenoids was 5.06, 3.23, 10.11, and 6.86 minutes, respectively. For the in vivo test, 20 (T1) and 30 g L^{-1} (T2), 0.5 (T3), and 0.8 ml ml^{-1} (T4) were used. Compared with each control treatment ~~control~~, all treatments used showed a reduction in their parasitological indices. Regarding ~~to~~ the effectiveness of each treatment, 29.9% was recorded for T1, 63.43% for T2, 99.81 for T, and 99.85 for T4. Fish exposed to different concentrations of salt and formalin did not show lethargy of the hypoxia signs. No behavioral changes were observed and all fish (100%) survived during the experiment. Thus, this study demonstrated the efficacy of using salt and formalin in the four concentrations tested.

Keywords: *Blackfin pacu, ectoparasites, formalin, Monogenoidea, salt.*

RESUMO

Colossoma macropomum é uma das principais espécies de peixes cultivadas na Amazônia peruana, sendo altamente demandada e aceita pela população local. Dentre os patógenos que parasitam peixes de água doce, destacam-se as espécies de Monogenoidea. Desta forma, o seguinte estudo teve como objetivo avaliar a eficácia do cloreto de sódio (sal) e formalina como tratamento contra monogenóides presentes em C. macropomum cultivado na Amazônia peruana. As espécies de Monogenoidea identificadas neste estudo foram Anacanthorus spatulathus, A. penilabiatus e Notozothecium janauachaensis. Testes *in vitro* e *in vivo* foram realizados no "Laboratorio de Parasitología y Sanidad Acuícola" localizado em Iquitos, Peru. Em relação ao ensaio *in vitro*, foram testados 20 e 30 mg ml $^{-1}$ de sal e 0,005 e 0,008 ml ml $^{-1}$ de formalina. O tempo médio de sobrevivência dos monogenóides foi de 5,06, 3,23, 10,11 e 6,86 minutos, respectivamente. Para o teste *in vivo*, foram utilizados 20 (T1) e 30 g L^{-1} (T2), 0,5 (T3) e 0,8 ml ml^{-1} (T4). Comparados com cada tratamento controle, todos os tratamentos utilizados apresentaram redução em seus índices parasitológicos. Quanto à eficácia de cada tratamento 29,9% foi registrado para T1, 63,43% para T2, 99,81 para T3 e 99,85 para T4. Os peixes expostos a diferentes concentrações de sal e formalina não apresentaram letargia dos sinais de hipóxia. Não foram observadas alterações comportamentais e todos os peixes (100%) sobreviveram durante o experimento. Desta forma, este estudo demonstrou a eficácia de usar sal e formalina nas quatro concentrações testadas.

Palavras-chave: Tambaqui, ectoparasitas, formalina, Monogenoidea, sal.

Recebido: fev./2023.
Publicado: mar./2024.

INTRODUCTION

Aquaculture has emerged as an alternative to generate solutions to increase the food supply to the growing world population as fish are excellent sources of animal protein of high nutritional value (FAO, 2003). Moreover, aquaculture is an important activity for the development of the Peruvian Amazon, which is considered essential for human nutrition, income, and work. Thus, *Colossoma macropomum,* popularly known in Peru as "gamitana", is one of the main fish species farmed in the Peruvian Amazonia, being highly demanded and accepted by the local population (MENDOZA, 2011).

Farmed fish are susceptible to attack by infectious agents, which usually do not affect fish health. Thus, there is a balance between the host, the parasite, and the environment. However, when the conditions become unfavorable for the fish (sudden temperature change, oxygen deficiency, high density, toxic elements in the water, lack of food), this balance is broken, weakening the fish immune system. Therefore, fish may become susceptible to the attack of opportunists pathogens, causing serious damage and high mortality rates in farming systems (VARGAS *et al*., 2015).

Among the main pathogens that parasitize freshwater fish, species of Monogenoidea stand out (FLORES-CRESPO and CRESPO, 2003). These ectoparasites, which are present in the body and gills of marine and freshwater fish, exhibit high host specificity (WHITTINGTON *et al.,* 2000), and sometimes, under culture conditions, high parasite loads may result in problems for the aquaculture systems (VENMATHI MARAN *et al.,* 2014). Monogenoids are ectoparasites that cause severe lesions in farmed fish (MARTINS *et al.,* 2001; TAVARES-DIAS *et al.,* 2001), leading to serious respiratory problems (TAVARES-DIAS *et al*., 2001). Thus, they are considered obstacles to animal productivity in intensive fish farming.

High levels of infestation by monogenoids can cause lesions in the gills, such as laceration of the filaments, epithelium destruction, thicker gill margins, and open opercula with necrotic areas, generating anastomoses that obstruct the exchange of gases in the capillaries and thus, decreasing the respiratory function, which may cause death by suffocation (FLORES-CRESPO and CRESPO, 2003).

Sodium chloride (NaCL), commercially named as salt, is an accessible and available product that is safe for people and does not leave residues in fish meat, being used in many countries to prevent and control fish parasites. Salt is essential for the daily management of freshwater fish since it helps counteract management stress, restore osmoregulation, and improve general condition, among other uses and benefits (KUBITZA, 2016).

Using chemical products in aquaculture is a recurring activity to prevent and control fish parasites. Among these products, formalin is highlighted for its effectiveness and accessibility (ANDRADE-PORTO *et al.,* 2017). However, therapeutic concentrations are applied without considering the adequate exposure time, toxicity, tissue alterations, and residues accumulation in the fish muscle, as well as changes in the homeostasis of exposed animals (JUNG *et al.,* 2001). Therefore, studies on the efficacy of salt and formalin to control monogenoids and their effects on fish are required. For this reason, the following work aims to identify the species of Monogenoidea in *C. macropomum* cultivated in the Peruvian Amazonia and to evaluate the efficacy of sodium chloride (salt) and formalin as a treatment against this group of parasites.

Recebido: fev./2023.
Publicado: mar./2024.

MATERIAL AND METHODS

This study was carried out according to the principles adopted by "Instituto de Investigaciones de la Amazonía Peruana" (IIAP). It has sanitary and administrative permissions for working with live fish: R.D. n° 132-2014-GRL-DIREPRO, R.D. n° 217-2016-GRL-DIREPRO PTH-068-16-PEC- SANIPES.

Fish and acclimatization

Individuals of *C. macropomum* with 12±3 cm standard length were collected from a fish pond of "Instituto de Investigaciones de la Amazonía Peruana" and acclimated in a tank of 100 L with a continuous flow of water and constant aeration in the "Laboratorio de Parasitología y Sanidad Acuícola". During acclimation, fish were fed with a commercial extruded feed containing 40% crude protein, once a day. Daily, the organic matter was removed from the bottom of the tanks and 30% of the water was changed.

In vitro assays with salt and formalin against Monogenoids of *C. macropomum*

To test the sensitivity of monogenoids from *C. macropomum* to salt and formalin (Sigma-Aldrich 40%), four naturally parasitized *C. macropomum* with 12±3 cm standard length were euthanized by medullar section. The parasitized gills were removed to determine the exposure time required to kill the Monogenoids attached to the gills, regarding different concentrations of salt and formalin. The experimental design consisted of four treatments: 20 and 30 mg mL^{-1} salt and 0.005 and 0.008mL mL^{-1} formalin, with three replicates added in ~~a~~ Petri dishes with 10 mL of water.

Each branchial arch of *C. macropomum* naturally parasitized by monogenoids was placed separately in a Petri dish and immersed in the different concentrations of salt and formalin. Four dishes used as control groups were prepared with water from the breeding tank. With a stereomicroscope, ±15 Monogenoids were selected in each repetition. After submerging the branchial arches in the different concentrations of the tested products, the parasites were observed using the microscope at 1-minute intervals to determine the survival time of monogenoids exposed to salt and formalin. 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 (SOARES *et al*., 2017).

The parasitological indices, as described by Bush *et al*. (1997), and the anthelmintic efficacy of each treatment were calculated according to the following formula proposed by Zhang *et al*. (2014): $AE = [B - T] \times 100\% / B$, where AE is the anthelmintic efficacy, B is the mean number of recorded Monogenoids in the control group, and T is the mean number of surviving Monogenoids in the treatment group. The time it took to kill 100% of Monogenoids was recorded. Moreover, the treatment was considered effective when 100% parasite mortality was achieved within 30 minutes.

To determine if the concentrations of salt and formalin used in the *in vitro* tests can be tolerated by *C. macropomum*, twelve glass aquariums (three for treatment) with 10 fish each were used. The treatments were based on the addition of 20 and 30 g L^{-1} of salt and 0.5 and 0.8mL L^{-1} of formalin into the glass aquariums containing the fish, which were kept there for 1 hour. During that time, fish were monitored to register any anomaly in their behavior or death.

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Following the same concentrations of salt and formalin considered in the *in vitro* tests, therapeutic baths against monogenoids of *C. macropomum* were used.

In vivo* assays of salt and formalin against Monogenoids of *C. macropomum

Seventy-two (72) individuals of *C. macropomum* with 12±3cm standard length naturally parasitized by Monogenoids were randomly distributed into twelve glass aquariums of 20 L^{-1} with a static water system and constant aeration for 24 h.

The therapeutic baths of 30 minutes consisted of four treatments (20 and 30g l^{-1} salt and 0.5 and 0.8mL L l^{-1} formalin) with three repetitions each and three fish in each repetition, totaling nine fish per treatment and nine fish as the control group. All treatments were performed in parallel with the control group. Each product was applied for an exposure time of 30 minutes for five days. Then, the fish were transported to similar glass aquariums with cleaned water. This process was daily performed until completing the five days of the experiment. During the bath, the behavior of the fish was observed. Fish were not fed during the experiment.

After applying the products for five days, fish were euthanized by medullar section and their gills were excised, fixed with hot water (68 ºC), and conserved with 5% formalin. Samples were examined with a stereomicroscope to identify and quantify the Monogenoids. The parasite species identification was carried out by clarifying the individuals in Hoyer´s medium and studying the sclerotized structures (BOEGER and VIANNA 2006), which were photographed with a digital camera (Olympus Qcolor 5) connected to a phase contrast microscope (Olympus BX 51). The images were used to illustrate the main morphological characteristics of the parasite species.

The prevalence, intensity, and mean intensity and abundance of infestation were calculated as described by Bush *et al*. (1997). The effectiveness of salt and formalin was compared using the formula proposed by Onake *et al*. (2003), as follows: Ef = (Mcont – Mtrat/Mcont) *100, where Ef is the effectiveness, Mcont is the mean number of parasites recorded in the control treatment, and Mtrat is the mean number of parasites recorded in the treatments.

Statistical Analysis

The Shapiro-Wilk test was used to determine the normality of the data. The simple one-way ANOVA was applied to determine significant differences among the treatments. The acquired data was archived in Microsoft Excel data sheets. The confidence level used was $p>0.05$ and all the statistics tests were performed using the statistical package BioStat. 5.0.

RESULTS AND DISCUSSION

Before the experiment, 445 monogenoids were collected from the fish species. From the total number of parasites recorded, three species of Monogenoidea were identified: *Anacanthorus spathulatus* (Fig. 01); *A. penilabiatus* (Fig. 02), and *Notozothecium janauachaensis* (Fig. 03).

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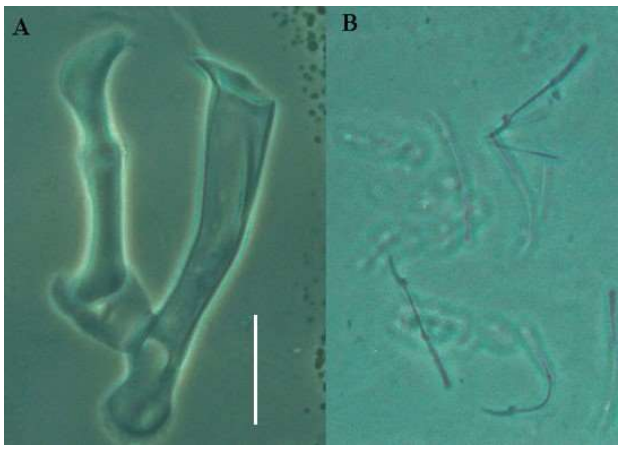
Anacanthorus spathulatus is characterized by presenting the copulatory complex in the form of "tweezer", elongated hooks with two subunits, thumb directed downwards, and short tip.



(Source: Personal archive, 2022**)**

Figure 01: Main sclerotized structures from *Anacanthorus spatulathus*.

Obs.: A = Copulatory complex; B = Hooks. Scale bar: 20µm.

Anacanthorus penilabiatus is characterized by presenting a tubular cirrus, sinuous in its middle part and with a projection directed downwards, forming a kind of "bird's beak". The accessory piece is rod-like, short, and straight. Hooks are short.

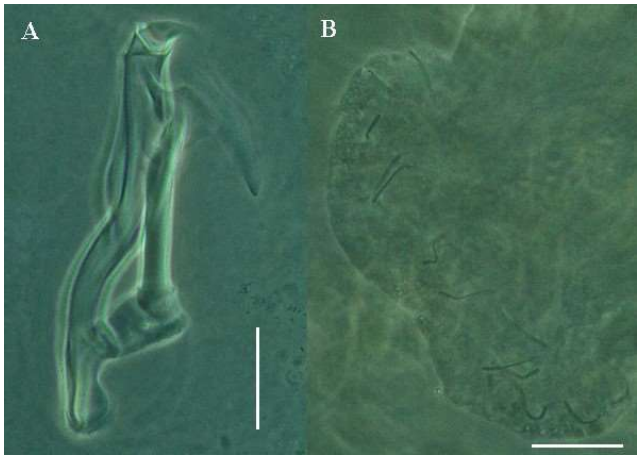


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Figure 02: Main sclerotized structures from *Anacanthorus penilabiatus*.

Obs.: A = Copulatory complex; B = Hooks. Obs.: Scale bar: 20µm.

Notozothecium janauchaensis is characterized by presenting a tubular cirrus, short, and in "comma-shaped". The accessory part is a thin band that articulates at the base of the cirrus. The ventral bar and anchors are larger than the dorsal ones. Moreover, the ventral bar is robust and elongated, while the dorsal bar is very thin.

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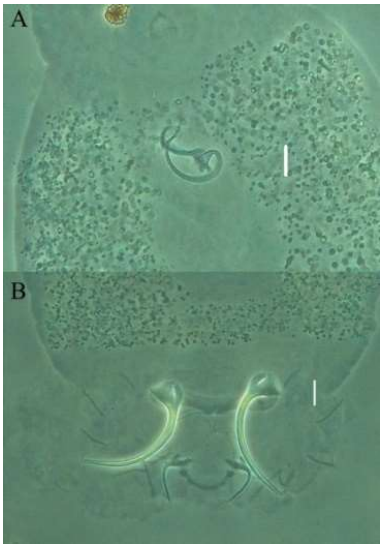

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Figure 03: Main sclerotized structures from *Notozothecium janauchaensis*.

Obs.: A = Copulatory complex; B = Haptor. Obs.: Scale bar: 10µm.

The total number of individuals from each treatment is shown in Tab. 01.

Table 01: Species and number of individuals of *Monogenoidea* found in the present study.

	Species of Monogenoidea		
Treatments	***Anacanthorus spathulatus***	***A. penilabiatus***	***Notozothecium janauachaensis***
20 g L $^{-1}$ salt	80	4	0
30 g L $^{-1}$ salt	163	6	1
0.5 ml l $^{-1}$ formalin	96	2	1
0.8 ml l $^{-1}$ formalin	87	3	2
Total number	**426**	**15**	**4**

The use of therapeutic baths, medicated rations, and the application of vaccines are practices usually applied to treat fish diseases (FOUZ *et al.,* 2001; WISE and TERHUNE, 2001). Common salt is used in prophylaxis baths since, in addition to reducing stress (URBINATI and CARNEIRO, 2004), it has proven its efficacy in disease prevention and treatments (PAVANELLI *et al.,* 2002).

Some studies have used sodium chloride as a preventive alternative against fish infectious pathogens. Salt and formalin are frequently applied incorrectly very late, being used at low and inefficient doses or for a short period. In Peru, studies regarding the use of these products in tropical fish to treat infestations caused by parasites, mainly monogenoids, are almost non-existent (KUBITZA, 2016).

In vitro tests showed the survival time of Monogenoids exposed to two different concentrations of salt and formalin. Results are presented in Tab. 02.

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Table 02: Mean survival time (minutes) of monogenoids present in the gills of *Colossoma macropomum* analyzed after the application of two concentrations of salt and formalin.

In vitro tests	20mg mL^{-1} salt	30mg mL^{-1} salt	0.005mL mL^{-1} Formalin	0.008mL mL^{-1} formalin
	Mean survival time (minutes)	**Mean survival time** (minutes)	**Mean survival time** (minutes)	**Mean survival time** (minutes)
Sample 1	4.82	3.02	9.25	8.31
Sample 2	4.43	3.08	13.18	5.64
Sample 3	5.93	3.58	7.89	6.63
Mean time	5.06	3.23	10.11	6.86

Parasitological indices of the Monogenoids recorded in the present study for each treatment with its respective control are presented in Tab. 03.

Table 02. Parasitological indices of the monogenoids recorded for the four treatments used during the experiment.

Treatments	EF	PF	TP	P%	mI	mA
T. control	4	4	117	100	29.3	29.3
T1: 20 g L $^{-1}$ salt	9	7	82	77.8	11.7	9.1
T. control	9	9	3039	100	337.7	337.7
T2: 30 g L^{-1} salt	9	8	988	88.9	123.5	109.8
T. control	9	9	4844	100	538.2	538.2
T3: 0.5 mL L^{-1} formalin	9	6	9	66.7	1.5	1.0
T. control	9	9	2987	100	331.9	331.9
T4: 0.8 mL L $^{-1}$ formalin	4	2	2	50	1.0	0.5

EF = examined fish, PF = parasitized fish, TP = total number of parasites, P% = prevalence, mI = mean intensity of infestation, mA = mean abundance of infestation.

Vargas *et al.* (2003) recommended the use of therapeutic baths with 3% salt for 10 min, which was effective against monogenoids belonging to *Gyrodactylus.* Forwood *et al*. (2013) evaluated the efficacy of alternative chemical treatments against the monogenoid *Lepidotrema bidyana* in *Pomoxis anularis* "silver perch". The effectiveness was determined *in vitro,* with treatments of sodium chloride at 1 g/L for 60 minutes. According to Murrieta-Morey (2020), the treatment against the monogenoid *Dawestrema cycloancistrium* with salt at 30g/L for 30 minutes almost completely decreased the number of parasites.

Chagas *et al.* (2012) investigated the use of salt at 2, 4, 6, and 8g L^{-1} for 30, 60, and 120 minutes. However, these concentrations were not effective in reducing the monogenoids parasites of *C. macropomum*. On the other hand, the use of salt at 20 and 30g L^{1} showed effectiveness values of 29.9 and 63.43, respectively. In that way, it was demonstrated that higher salt concentrations are more effective against monogenoids.

Formalin, when administered at 30g L^{-1}, can also be used; however, vigorous aeration is required to maintain an acceptable level of dissolved oxygen. Sick fish do not tolerate formalin well. Thus, all animals should be carefully monitored during the chemical administration. If an adverse reaction is noticed, fish should be immediately removed from the

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treatment tank and placed in clean water. Formalin removes oxygen from the water. Therefore, ~~so~~ vigorous aeration must be provided. Moreover, formalin should never be applied to ponds at dusk (REED *et al.,* 2012).

Each product was compared with its respective control treatment, revealing effectiveness higher than 99% for both treatments with formalin. The values are presented in Tab. 04. The statistical analysis showed significative differences when comparing each treatment used with its respective control treatment: T1:T0 with p=0.0009; T2:T0 with p=0.0002; T3:T0 with p=<0.0001; T4:T0 with p=<0.0001. Fish exposed to different concentrations of salt and formalin did not exhibit lethargy of the hypoxia signs. No behavioral changes were observed, and all fish (100%) survived during the experiment.

Table 03: Effectiveness of each treatment used compared with its respective control treatment

Treatments	Mean of parasites	Effectiveness (%)
T. Control	29.25	
20 g l $^{-1}$ salt	9.11	29.9
T. Control	333.67	
30 g l $^{-1}$ salt	123.5	63.43
T. Control	538.22	
0.5 ml l $^{-1}$ formalin	1	99.81
T. Control	331.89	
0.8 ml l $^{-1}$ formalin	0.5	99.85

Andrade-Porto *et al.* (2018) reported 93.6% and 99.3% efficacy when using formalin at 440 and 550 mg L^{-1}, respectively, for 1 hour of bath, whereas longer (12 hours) resulted in lower efficacy. Similarly, ~~in~~ therapeutic baths with 250 mg L^{-1} formalin for 1 hour against *Benedenia seriolae* and *Zeuxapta seriolae* in *Seriola lalandi lalandi* showed 80% of efficacy (SHARP *et al.,* 2004). For *Hemigrammus* sp. exposed to 27.5 mg L^{-1} formalin for 1 hour, the efficacy was 77.7% (PAIXÃO *et al.,* 2013). In contrast, for *Haliotrema abaddon* in the gills of *Glaucosoma hebraicum* treated with 25 mg L-1 formalin for 10 and 24 hours, no antiparasitic effect was observed (STEPHENS *et al.,* 2003).

Twenty-four-hour baths with 27.5mg L^{-1} formalin, in six alternate-day applications, showed 71% efficacy in controlling monogeneans in *Pterophyllum scalare* (FUJIMOTO *et al.,* 2018). Nevertheless, as formaldehyde is a human carcinogen (LAI *et al.,* 2016) and a toxic product, formalin should be handled carefully during antiparasitic treatments in fish farming (ANDRADE-PORTO *et al.,* 2018)

Formalin may cause tissue changes in fish gills, which can lead to osmoregulatory disorders (ANDRADE-PORTO *et al.,* 2017). In general, formalin may affect the gills, acting on the ionic regulation that induces a rapid decrease in plasma electrolyte levels (JUNG *et al.,* 2001).

Studies have suggested that using formalin in long baths to control ectoparasites in fish is less effective than in short ones, as it is rapidly consumed within the water column (STEPHENS *et al.,* 2003).

The *in vitro* and *in vivo* results showed that the antiparasitic efficacy of formalin against *D. cycloancistrium* was dose-dependent. Short baths (1 hour) with concentrations of

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440 and 550mg L^{-1} formalin are recommended to control *D. cycloancistrium* in *A. gigas* gills without compromising fish homeostasis. However, caution is advised regarding the use of such concentrations directly into farming tanks, since synergistic and antagonistic effects may occur due to variations in different environments.

Mortality of *C. macropomum* was not observed during the therapeutic baths with salt and formalin, since the lethal concentration for the species was previously determined. In this study, the effectiveness of using salt and formalin at the four concentrations tested was demonstrated. Formalin at 0.5mL L^{-1} and 0.8mL L^{-1} showed the best results, with values of effectiveness higher than 99%. However, future studies should be carried out to evaluate other fish species, which may present different degrees of susceptibility, as well as other concentrations of salt, formalin, and also natural products (phytotherapy).

CONCLUSIONS

Monogenoidea species that parasitize the gills of *C. macropomum* from fish ponds located in IIAP include: *A. spathulatus, A. penilabiatus,* and *N. janauachaensis*. However, salt and formalin can be used to control these monogenoids. Two doses of formalin (0.5mL l^{-1} and 0.8mL l^{-1}) showed an efficacy greater than 99%, eliminating practically all the monogenoids presented in the *C. macropomum* gills. Moreover, the fish did not show signs or symptoms of damage when treated with the doses used in this study, obtaining ~~a~~ 100% survival for all the treatments evaluated.

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Recebido: fev./2023.
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Recebido: fev./2023.
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