Aquaculture 470 (2017) 123–128

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Levamisole reduces parasitic infection in juvenile pacu (*Piaractus mesopotamicus*)

Eduardo Pahor-Filho [a,*], Joaber Pereira Júnior [c], Fabiana Pilarski [a], Elisabeth Criscuolo Urbinati [a,b]

[a] Centro de Aquicultura, Universidade Estadual Paulista - UNESP (CAUNESP), 14.884-900 Jaboticabal, São Paulo, Brazil
[b] Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista – UNESP, 14.884-900 Jaboticabal, São Paulo, Brazil
[c] Universidade Federal do Rio Grande - FURG, 96200-400, Rio Grande, Rio Grande do Sul, Brazil

ARTICLE INFO

Article history:
Received 12 November 2016
Received in revised form 13 December 2016
Accepted 18 December 2016
Available online 28 December 2016

Keywords:
Anthelmintic
Aquaculture
Imidotiazol
Liver histology
Parasites

ABSTRACT

Anthelmintic drugs are successfully used in aquaculture to control parasitic infections or infestations. This study analyzed the effectiveness of levamisole as an antiparasitic and its effect on the liver of juvenile pacu (*Piaractus mesopotamicus*). A total of 300 fish (180 ± 1.27 g; 16 ± 0.4 cm) were fed a diet containing levamisole hydrochloride (LHC) for fifteen days. A control group (T0) and four treatments were tested: T1 (100), T2 (150), T3 (300) and T4 (500) mg kg^{-1} LHC in quadruplicate (15 fish per repetition). Four fish per treatment (n = 20) were euthanized by sectioning the spinal cord; the sample was forwarded for parasitological analysis. The gill arches and intestines were removed and preserved in formaldehyde 10% for identification and quantification of the parasites. For evaluation of the liver histology, four fish per treatment (n = 20) were euthanized with benzocaine (100 mg L^{-1}) for liver removal. In intestines and gills, we identified the parasites *Rondonia rondoni* (Nematoda, Atractinae) and *Anacanthorus penilabiatus* (Monogenoidea, Dactylogyridae), respectively. Both were at high prevalence and intensity of infection and infestation, respectively. The 300 mg kg^{-1} LHC treatment reduced the infection by *R. rondoni* but not the infestation by *A. penilabiatus*. Concentrations of 150 and 300 mg kg^{-1} LHC caused moderate liver changes, and no changes were observed in fish treated with 100 mg kg^{-1} LHC. The highest concentration of LHC (500 mg kg^{-1}) induced a high occurrence of sinusoid dilation, blood congestion and leukocyte infiltration in fish liver. Our results indicate that LHC (300 mg kg^{-1}) was effective in controlling the infection by *R. rondoni* and caused moderate histological changes in pacu liver.

1. Introduction

Parasitosis in aquaculture causes damages in the host and predisposes it to secondary infections resulting in significant mortalities (Ogawa, 2015). This motivates the development of new therapeutic treatments of parasitic control (Alvarez-Pellitero, 2008). Infection by Nematoda (Campos et al., 2009) and infestation by Monogenoidea (Jorgensen et al., 2009) have been recorded frequently in fish farming. The identification and quantification of these parasites in important farmed fish are necessary tools to control parasitic diseases.

Parasitic control in fish can be effectively performed by chemotherapeutic drugs. The levamisole hydrochloride (1-2-3,5,6-tetrahydro-6-phenylimidazol [2,1-b] thiazole) is an anthelmintic belonging to the imidotiazol group that is effective in controlling parasites in humans (Harhay et al., 2010) and other animals (Cruz et al., 2010; Idika et al., 2012). It immobilizes the parasite by muscle paralysis (Martin et al., 2012) and is an alternative treatment for nematodes (Geets et al., 1992) and protozoa (Findlay et al., 2000). In fish, it had positive effect against parasites (Martin et al., 2012; Findlay et al., 2000) and bacteria (Hang et al., 2014.). This drug has low cost and can be readily absorbed by the body (2–3h) such as levamisole hydrochloride (LHC). It has a short plasma half-life of 4 h, and its removal occurs in the first 12 h (40% in urine and 41% in excreta).

LHC powder is commercially available and can be administered orally to fish (Gopalakannan and Arul, 2006; Biller-Takahashi et al., 2013) as well as by intraperitoneal injection (Hang et al., 2014) and by prophylactic baths (Findlay et al., 2000). This drug has a remarkable immunostimulating effect when added to the diet (Mulero et al., 1998; Gopalakannan and Arul, 2006; Ispir and Yonar, 2007; Maqsood et al., 2009; Sado et al., 2010; Biller-Takahashi et al., 2013; Hang et al., 2014; Biller-Takahashi et al., 2016). Thus, the immunomodulatory activity of the levamisole may be an important strategy to reduce stress, making fish more resistant to pathogenic infections (Biller-Takahashi et al., 2016).

Inclusion of anthelmintics in the diet is an alternative to avoid deleterious effects of the parasites and may increase the chance of fish survival (Barman et al., 2013). However, the effective concentration of an antiparasitic varies according to the stage development and species of fish (Oliva-Teles, 2012). Thereafter, it is necessary to know the safe

* Corresponding author.
E-mail address: efpahor@gmail.com (E. Pahor-Filho).

http://dx.doi.org/10.1016/j.aquaculture.2016.12.034
0044-8486/

concentration of the antiparasitic because the excess can cause histopathological effects (Morrison et al., 2001) and even fish death (Pahor-Filho et al., 2014).

Pacu, *Piaractus mesopotamicus* (Holmberg, 1887), is an important sport and table fish in South America (Oliveira et al., 2004; Queiroz et al., 2005) that is farmed in aquaculture systems (Oliveira et al., 2004; Queiroz et al., 2005; Urbinati et al., 2013; Valladão et al., 2016). Its parasitic fauna is known (Costa, 1963; Boeger et al., 1995; Martins and Urbinati, 1993; Tavares-Dias et al., 2001; Martins and Onaka, 2004; Campos et al., 2009; Campos et al., 2011; Franceschini et al., 2013). It is common the reports of mortality of pacu affected by these parasites (nematodes and monogeneans) in the Brazilian fish farms. Nematoda are light-colored roundworms, cause intestinal lumen obstruction and often compete for food in the host's intestines (Buchmann and Lindenstrom, 2002). Monogenoidea are ectoparasitic flatworms that are small and generally more aggressive than nematodes and cause epithelial and hematological alterations (Martins and Urbinati, 1993; Araújo et al., 2009). In this way, the development of the new parasitic control techniques is essential to avoid mortality and economic loss in pacu farming.

In order to establish a parasitic control model, this study identified and quantified parasites in pacu and examined the effectiveness of LHC as an antiparasitic as well as the effects of its oral administration on the liver.

2. Material and methods

All experimental procedures that involved animals were performed in accordance with the ethical principles in animal experimentation adopted by the Colégio Brasileiro de Experimentação (COBEA) Brasilia, Brazil, and approved by the Ethics and Animal Welfare Committee (CEUA) of the School of Agricultural Sciences and Veterinary Medicine of São Paulo State University (UNESP), Jaboticabal, SP, Brazil – protocol no 002214/12.

2.1. Design and experimental protocol

A total of 300 juvenile pacu (180 ± 1.27 g, 16 ± 0.4 cm) obtained from a commercial fish farm were distributed in 20,125-L fiber tanks in a water recirculation system. The photoperiod was 12 h light:12 h dark. Fish were fed daily with an experimental diet (3% of the body weight) twice daily (8:30 AM and 17:00 PM) for 15 days. A control group (T0) and four treatments: T1 (100), T2 (150), T3 (300) and T4 (500) mg kg^{-1} LHC (15 fish per repetition) were tested in quadruplicate. The LHC concentrations were chosen according to Biller-Takahashi et al. (2013).

During the experimental period the water quality parameters remained in an acceptable range for tropical fish (Vinatea-Arana, 2003) with the following values: temperature (28 ± 0.16 °C), pH (7.46 ± 0.14), oxygen level (6.43 ± 0.86 mg L^{-1}) and total ammonia (0.21 ± 0.03 mg L^{-1}).

2.2. Oral administration of LHC and analysis

Before the experimental period, fish were acclimatized in the tanks, in a recirculation system, for fifteen days. During this period, they consistently fed a diet without levamisole and presented good health status without clinical signs of disease. After this, a total of 60 fish were sampled to evaluate the parasitic infestation and infection and liver histology. Two fish from each tank (n = 40) were euthanized by section of the medullae to avoid interference in the parasitological indexes (Eiras et al., 2006). Gills and intestines were forwarded to parasitological analysis. For histological analysis, one fish per tank (n = 20) were euthanized with benzocaine (100 mg L^{-1}), and the liver was removed.

2.3. Parasitological analysis of the gills and intestines

Gill arches and intestines were fixed in 10% formaldehyde. The preparation of the helminth parasites was performed according to Amato et al. (1991). All gill arches on one side of the fish were harvested and deposited into petri dishes for parasitological quantification under stereomicroscope. After this, parasites were collected from gills, preserved in 5% formaldehyde for 24 h and transferred to 70% alcohol. Then they were dehydrated in an ethyl alcohol sequence between 70 and 100%, stained with Masson trichrome and mounted in Grey-Wess' medium to check sclerotized parts (haptor and copulatory organs).

Endoparasites were fixed in AFA for 24 h and then preserved in 70% alcohol, cleared in Amann Lactophenol and mounted in Canada balsam to check copulatory organs and esophagus.

The way in which the parasites use the host was evaluated by calculating the parasitological indexes of prevalence (P%), intensity of infection or infestation (II), average intensity of infection or infestation (IMI) and average abundance (AX) according to Bush et al. (1997).

The *Rondonia rondoni* (Nematoda: Atractinae) were identified in intestines according to Costa (1963) and the *Anacanthorus penilabiatus* (Monogenoidea: Ancyrocephalinae) in gills according to Boeger et al. (1995).

2.4. Histological effects of LHC in the liver

Tissue samples from all groups were fixed in Bouin solution during 4 h and then maintained in 70% ethyl alcohol and embedded in Paraplast® (Sigma-Brazil). Histological sections (5 μm) were prepared and stained with hematoxylin and eosin. The samples were analyzed using optical LEICA DM2500 microscope.

2.5. Experimental diets

Different concentrations of LHC (IMEVE, http://www.imeve.com.br/azul/) were added to the feed (FRI-AQUA® omnivorous fish, 26% crude protein and 3200 kcal metabolizable energy) previously ground. The feeds were homogenized with addition of 30% water, and the pellets were made in a meat grinder and sun dried. The finished diets were stored at −20 °C.

2.6. Statistical analysis

The experiment was set up in a completely randomized design. The results of efficacy of LHC (parasitic infection or infestation) were studied with a two-way analysis of variance (ANOVA, $\alpha = 0.05$) followed by the Tukey test to check for significant differences between treatments using the SAS statistical program. The comparison between the parasitological indexes was performed by parasitological statistical analysis protocol using quantitative parasitology according to Reiczigel and Rózsa (2005). The results were analyzed by the Kolmogorov-Smirnov test, to determine the normality of data. The Z test was performed to verify the difference of prevalence between the treatments. The nonparametric Kruskal-Wallis test followed by the Dunn method was performed to compare the abundance and mean intensity in the different treatments ($p < 0.05$).

3. Results

3.1. Parasitological indexes

The type of host (pacu) parasitism was determined by calculating the parasitological indexes. The control fish presented high prevalence and intensity of infection and Infestation by both parasites species. The fish treated with high LHC concentrations presented lower prevalence and intensity of infection by endoparasites (Table 1).

Table 1
Parasitological indexes of parasitic infection or infestation in pacu. T = treatments, T1 = 100, T2 = 150, T3 = 300, T4 = 500 mg kg^{-1} of levamisole hydrochloride, and T0 = control (n = 40). P (%) – prevalence, II – intensity of infection or infestation, AII – average intensity of infection or infestation and AX – average abundance.

Parasites	Treatments	P	II	AII	AX
R. rondoni	T1	100	216–237	226.25 ± 15.04	45.25 ± 6,72
A. penilabiatus		100	8–80	43 ± 6.55	8,6 ± 2.93
R. rondoni	T2	100	100–178	129.87 ± 11.39	25.97 ± 5.09
A. penilabiatus		100	20–56	36 ± 6	7,2 ± 2.68
R. rondoni	T3	50	5	1.62 ± 1.27	0.32 ± 0.56
A. penilabiatus		100	26–66	90 ± 9,48	9 ± 3
R. rondoni	T4	–	–	–	0
A. penilabiatus		87.5	96	–	8.1 ± 2.84
R. rondoni	T0	100	230–420	316.25 ± 17.78	63.25 ± 7.95
A. penilabiatus		100	20–98	53 ± 7.28	10.6 ± 3.25

3.2. Effectiveness of levamisole hydrochloride in parasitic control

The effectiveness of the LHC concentration was quantified in parasitic control. The increase in the dietary LHC concentration decreased prevalence and intensity of infection by *R. rondoni* (Fig. 1). The infestation by *A. penilabiatus* not was influenced by LHC concentrations (Fig. 2).

3.3. Identification of morphological and histological changes in the liver

The morphological and histological changes observed in the liver of pacu fed with LHC are shown. Fish from T0 and T1 treatments presented cordonal arrangement of hepatocytes and liver with normal appearance (Fig. 3A and B). In T2 group, fish showed cordonal arrangement of hepatocytes and 40% presented dilation of sinusoid (Fig. 3C). In T3 group, fish showed cordonal arrangement of hepatocytes, 50% showed dilation of sinusoid and 40% presented congestion and leukocyte infiltrate (Fig. 3D). In T4 group, fish showed cordonal arrangement of hepatocyte, sinusoid dilatation, congestion and leukocyte infiltrate (Fig. 3). Fish presented few degenerate hepatocytes in all treatments, except in control group. There was overall survival of fish from control group and LHC treatments except T5 fish whose survival was 80%.

3.4. Quantification of morphological and histological changes in the liver

The quantification of morphological and histological changes observed in the liver of pacu fed with LHC is shown. The fish from control group presented 100% of frequency of histological and morphological changes. The increase of levamisole concentration induced a higher frequency of histological changes in the liver of fish treated with LHC (Table 2).

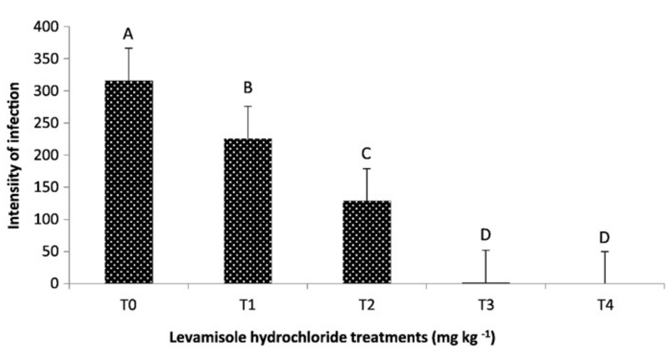

Fig. 1. Infection by *Rondonia rondoni* in pacu (n = 40) fed during 15 days with levamisole hydrochloride: T0 (control), T1 (100 mg kg^{-1}), T2 (150 mg kg^{-1}), T3 (300 mg kg^{-1}), or T4 (500 mg kg^{-1}). Capital letters indicate differences between treatments by Tukey test ($p < 0.05$).

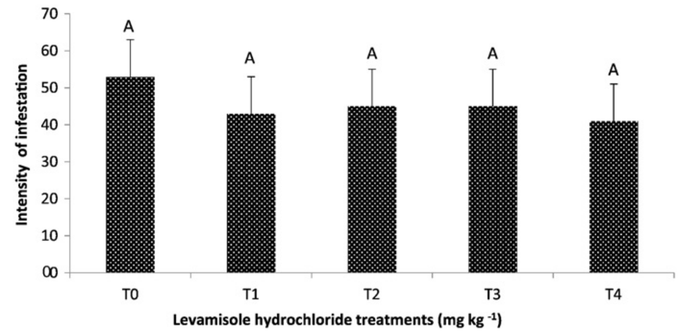

Fig. 2. Infestation by *Anacanthorus penilabiatus* in pacu (n = 40) fed during 15 days with levamisole hydrochloride: T0 (control), T1 (100 mg kg^{-1}), T2 (150 mg kg^{-1}), T3 (300 mg kg^{-1}), or T4 (500 mg kg^{-1}). Capital letters indicate differences between treatments by Tukey test ($p < 0.05$).

4. Discussion

In the present study, we analyzed the effectiveness of levamisole as an antiparasitic and the liver changes induced by its oral administration in pacu. We evaluated parasitological indicators, quantification and parasitic control and histological analysis of the liver. The results indicated efficacy of LHC in controlling the infection by *R. rondoni* but not the infestation by *A. penilabiatus*. LHC concentrations below 300 mg induced fewer changes in fish liver, which suggests that these changes might be reversible.

In aquaculture, parasitosis can cause large mortalities (Kristmundsson and Helgason, 2002) and parasitic control is an alternative to avoid losses (Martin et al., 2012). Among the parasites, nematodes can infect fish in the muscles, abdominal cavity and especially in the intestines. Their pathogenicity depends on the host species, infected organ and intensity of parasitosis (Tatcher, 1991). Infection by *R. rondoni* in pacu has been reported (Costa, 1963; Martins and Urbinati, 1993; Campos et al., 2009). The occurrence of diseases associated with *R. rondoni* infestation is not common (Tatcher, 1991) even with record high infection intensities (675-49476), however nematodes can cause obstruction of the intestinal lumen at a high intensity of infection (Campos et al., 2009). There are several reports of mortality of pacu affected by these parasites (nematodes and monogeneans) in the Brazilian fish farms. In this line, there is a justification to carry out the parasitic control in the pacu aquaculture, as a way to avoid suppression of the productive performance and mortalities.

There are cases in rearing systems, in which the high prevalence (89.3%) and intensity of infection by *R. rondoni* did not cause death of the pacu (Franceschini et al., 2013). In our study, we observed the prevalence (100%) and intensity of infection (230–420) by nematodes in control fish and various infective stages (larvae, juveniles, adults, ovigerous females and with larvae) but again with no mortality. The high prevalence and intensity of infection by *R. rondoni* in pacu can be explained by endogenic multiplication of the parasite in fish and the specificity of this nematode by the host as postulated by Campos et al. (2009).

Monogenoidea also occur in fish with high prevalence and intensity of infestation in the nature (Pahor-Filho et al., 2012) or in rearing systems (Franceschini et al., 2013). However, in contrast to what we observed with *R. rondoni*, many species are pathogenic and can cause severe injuries in the gills (Arafa et al., 2009; Dezfuli et al., 2007) and even death of the host (Kristmundsson and Helgason, 2002). Pacu can be infested by *A. penilabiatus* (Boeger et al., 1995; Franceschini et al., 2013) and *Mymarothecium viatorum* (Cohen and Kohn, 2005) both Monogenoidea. Ecological relationships among the parasites of this group and fish suggest that Monogenoidea also have high specificity for their host (Buchmann and Lindenstrom, 2002; Cribb et al., 2002).

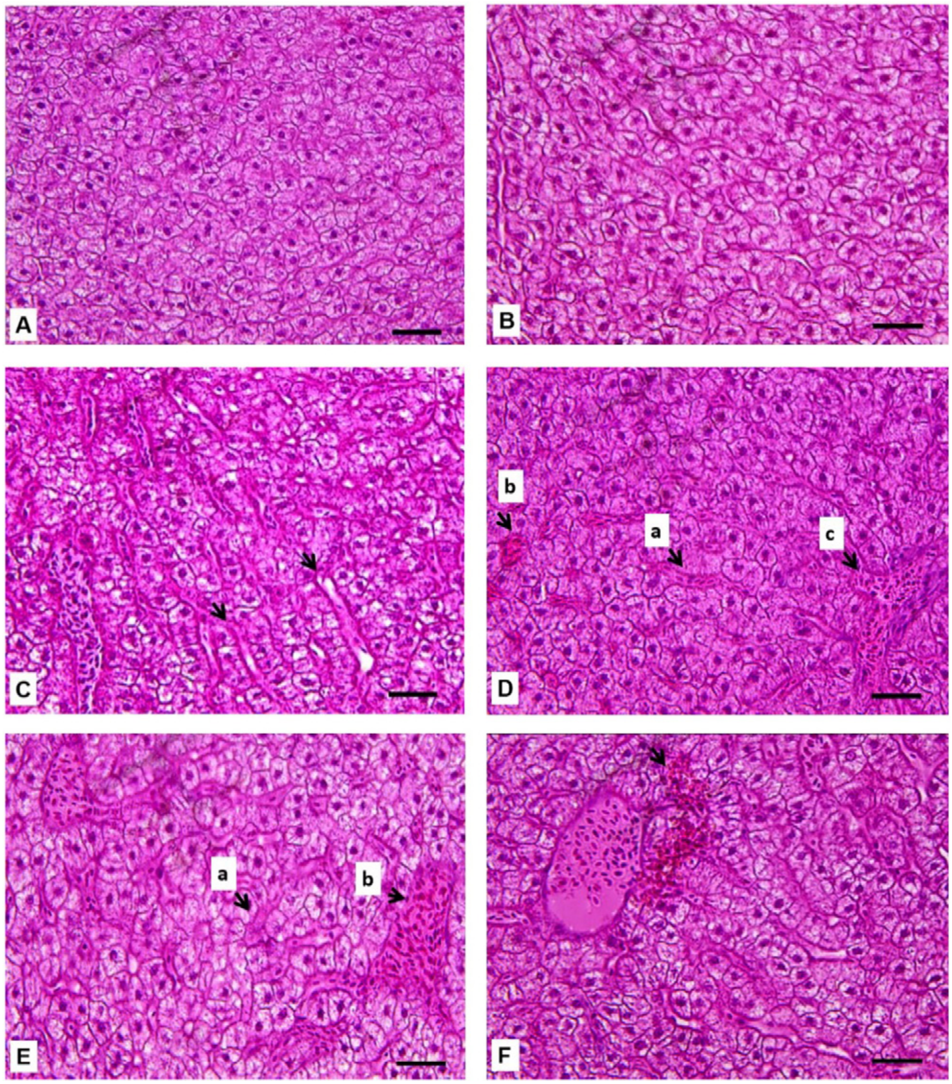

Fig. 3. Photomicrograph of the liver of pacu (n = 20) fed during 15 days with levamisole hydrochloride: T0 (control), T1 (100 mg kg^{-1}), T2 (150 mg kg^{-1}), T3 (300 mg kg^{-1}), and T4 (500 mg kg^{-1}). Panel A (control): cordonal arrangement of hepatocytes and liver without histological changes. Panel B (T1): cordonal arrangement of hepatocytes, liver without histological changes. Panel C (T2): (arrow): dilation of sinusoid. Panel D (T3): (a) dilation of the sinusoid; (b) congestion and (c) leukocyte infiltrate. Panel E (T4): (a) dilation of the sinusoid and (b) infiltrate leucocyte. Panel F (T4) (arrow): congestion. Hematoxylin and eosin staining (40×). Scale bar = 100 μm.

Herein, we observed an *A. penilabiatus* prevalence of 100% in pacu with an intensity of infestation of 20–98. This result can be explained by the monoxenous life cycle of Monogenoidea and the contact of fish in the rearing system used factors that certainly increase the horizontal parasitic transmission. According to Jorgensen et al. (2009), these parasites can cause severe injuries to fish due to aggression caused by haptor in the gills. However, in our study, we did not observe fish mortality associated with parasitism by *R. rondoni* and *A. penilabiatus* even in high prevalence and intensity of infection or infestation, respectively. It is likely that levamisole feeding may have improved resistance of fish to parasitic infection and infestation. Reports in the literature show that levamisole improved immune system of fish (Mulero et al., 1998; Gopalakannan and Arul, 2006; Hang et al., 2014; Biller-Takahashi et al., 2016), however, in this study we did not perform immune analyzes.

Table 2
Morphological and histological changes (%) in liver of the pacu fed with diets with levamisole hydrochloride. T = treatment, T1 = 100, T2 = 150, T3 = 300, T4 = 500 mg kg^{-1} of levamisole hydrochloride, and T0 = control (n = 20). CAH = cordonal arrangement of hepatocytes, DS = dilation of sinusoid, CO = congestion and LI = leukocyte infiltrate.

T	mg kg^{-1}	Morphological characteristics and histological changes (%)			
		CAH	DS	CO	LI
T0	0	100	0	0	0
T1	100	100	0	0	0
T2	150	100	40	0	0
T3	300	100	50	40	40
T4	500	100	100	100	100

Anthelmintics administered in the feed or water may control the infection by parasites and bacteria (Sakai, 1999). Among them, levamisole is effective in controlling the protozoan (Findlay et al., 2000) and nematodes (Geets et al., 1992). LHC (300 and 500 mg kg^{-1}) consistently controlled the infection by *R. rondoni*, but not the infestation by *A. penilabiatus*, showing its anthelmintic effect in pacu. It is possible that the blocking action of receptors of the cholinergic ion channels (AChRs), stimulated by LHC, has no effect on ectoparasites. This occurs due to the specificity of the gastrointestinal nematodes by cholinergic tissue as postulated by Martin et al. (2012). On the other hand, LHC was administered in the diet and not in water, which may have avoided contact of the anthelminthic with ectoparasites, making parasitic control of Monogenoidea difficult.

The metabolic reactions of chemotherapy can cause histological injuries in liver (Roberts, 2001). Therefore, it is critical to find a concentration of the drug that is safe and effective without intoxicating the fish. In the current study, except in the control group, fish treated with levamisole showed few degenerate hepatocytes, indicating an effect of the drug in liver. Herein, the highest dietary LHC concentration (500 mg kg^{-1}) caused higher frequency of the sinusoid dilatation, congestion and leukocytic infiltration in pacu indicating the effort of the liver to metabolize a high concentration of LHC with toxic effects. However, at lower concentrations, LHC (300 mg kg^{-1}) caused a lower frequency of changes in the liver. In another study, juvenile salmon (*Salmo salar*) treated with levamisole in the water (0.5 μg g fish^{-1}) and with intraperitoneal injection (0.1 ml fish^{-1}) presented a significant increase in mucous cells and lamellar fusion of the gills after four weeks of treatment compared to control fish. The authors suggested that the increase in mucous cells could be related to the fish effort to minimize the gill changes caused by levamisole (Morrison et al., 2001).

In this study, LHC (300 mg kg^{-1}) was effective in controlling the infection by *R. rondoni* and caused a lower frequency of liver changes in pacu. These results suggest that this concentration was safe and effective for parasitic control. There was 100% survival during the feeding period with LHC. According to Roberts (2001), the liver has an important role to metabolize substances and detoxify the body. It is an organ that is vulnerable to the histological changes caused by toxic substances. Our data suggest an alternative use of the LHC (300 mg kg^{-1}) as an antiparasitic agent in rearing systems of the pacu.

Our study contributes to the development of new therapeutic treatment in the farming of pacu by oral administration of levamisole. Further studies should advance this work to other farmed species, other stages of fish development and period of the administration and action of the drug.

Acknowledgment

The authors thank the Aquaculture Center at UNESP for the supply of fish and CAPES for the scholarship provided to the first author.

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