Latin American Journal of Aquatic Research, 47(1): 34-41, 2019
DOI: 10.3856/vol47-issue1-fulltext-5

Research Article

Formalin toxicity to *Oreochromis niloticus*; its effectiveness against C*ichlidogyrus* spp. and host stress response

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ABSTRACT. The effective formalin therapeutic concentration (ETC) against *Cichlidogyrus* spp. infecting tilapia was obtained. Also, the stress and immune response to formalin baths in *O. niloticus* were achieved. A static bioassay with four concentrations (0.5, 1.0, 1.5, 2.0 mL L^{-1}) of formaldehyde (37% dilution) in water, and one control without this substance was performed. Results evidenced that 1.5 mL L^{-1} of formalin for 40 min was the ETC to eradicate monogeneans without manifesting macroscopic damage to fishes. Thirty tilapias underexposure with ETC of formalin (FORM Group) were evaluated for stress, utilizing cortisol and glucose determination, and immune response (lysozyme activity and differential counting of organic defense cells). Other 30 tilapias without cichlidogyriasis and with no exposure to formalin were the Control Group. Evaluation times were: three hour pre-treatment (T0), and one hour, three days and three months post-treatment (T1, T2, and T3, respectively). A peak of cortisol (~326 ng mL^{-1}) was observed in tilapias of FORM Group at T1 with significant differences with the control group ($P < 0.05$). Similarly, glucose concentration reached significant values (~119 mg dL^{-1}) at one hour after the conclusion of treatment ($P < 0.05$). Those values returned to the basal limit at the last time of the study. The lysozyme activity and white blood cells in differential counting showed no significant differences in the same conditions ($P > 0.05$). Results confirmed total control of cichlidogyriasis with the ETC of formalin obtained and an evident resistance of tilapia to treatment as stabilization of its physiological stress response was achieved.

Keywords: tilapia; *Cichlidogyrus*; monogeneans; serum cortisol; serum glucose; innate response

INTRODUCTION

Tilapia culture has increased worldwide in the last decade with a production of about 3 million tons that equals approximately US$ 5,000 million (FAO, 2014). This fish is also the most cultivated in Mexico, with an increasing value market of US$ 475 million per year (CONAPESCA, 2013). In particular, in the Yucatan State, located in southeastern Mexico, tilapia production has a continuous range of 140 ton per year since 2012 (Flores-Nava *et al*., 2016).

Tilapia farming provides an alternative to cheap animal protein and a source of employment. However, consecutive losses due to fish mortalities may promote either their closure or owners could change the productive activity (Toledo *et al*., 2008). Therefore, it is necessary to improve the routine sanitary procedures with the objective of preventing pathologies. For example, those caused by mono-geneans parasites can produce external abrasions in fishes contributing to the presence of secondary infections, causing severe reduction of the production profitability by associated mortalities (Vidal-Martínez *et al*., 2002; Gonzales-Fernández, 2012).

In Mexico and other countries, monogeneans have been registered in farmed tilapia; *Cichlidogyrus sclerosus* is one of the most prevalent ectoparasitic monogeneans of this genus (Vidal-Martínez *et al*., 2002;

Corresponding editor: Alvaro Bicudo

Sandoval-Gío *et al*., 2008, 2013; Mendlová *et al*., 2012; Salgado-Maldonado & Rubio-Godoy, 2014). In Yucatan, *C. sclerosus* is the most prevalent monogenean in tilapia facilities (Paredes-Trujillo *et al*., 2016).

Formaldehyde (formalin) is a chemical compound (CHO) that can be used as antiparasitic control in fish culture (Leal *et al*., 2016). Nowadays, the Food and Drug Administration (FDA) has approved and recommended three commercial products with formalin as an active principle for use in US aquaculture, especially for the eradication of monogenean infestations (*i.e.*, *Cleidodiscus*, *Dactylogyrus*, *Gyrodactylus* spp.) (Nasser *et al*., 2017). Likewise, in Mexico, some studies have registered the effectiveness of formalin to control ectoparasites, as monogeneans, in cultivated fish (Jiménez-Guzmán *et al*., 1988; Sandoval-Gío *et al*., 2008).

It is necessary to point out that the supply of several substances to water, *i.e.*, therapeutants, is a common practice in aquaculture that produces an alteration in the physical and chemical composition of aquatic environment of fishes and then a stress response could be achieved (Kubilay & Ulukoy, 2002).

In the initial stages of intoxication derived by addition of chemicals to water, the physiological changes that take place in fish can be measured using serum indicators, where the cortisol is the most widely known (Cnaani *et al*., 2004; Ellis *et al*., 2012). Cortisol is a hormone secreted by the hypothalamic-pituitary-interrenal axis and increases of levels in organisms is a sign of primary stress response, while a rise of glucose levels in plasma or serum indicates a secondary stress response (Sanches *et al*., 2015).

Furthermore, external stressors, as the chemotherapeutic treatments, can induce variations in teleost's immune parameters (Holladay *et al*., 2010). In this case, changes in levels of lysozyme could be a general parameter of determination of external alteration in cultured fishes (Girón-Pérez *et al*., 2009; Ahmadi *et al*., 2014). However, specific data about both stress response and potential immunosuppression of formalin in tilapia are not defined; consequently, it is necessary to determine the tolerance to this substance in this species. In this sense, because formalin has chemical properties that make it appropriate to produce possible immunotoxic effects on fishes under therapeutic procedures, this study aimed to evaluate stress and innate immune response in tilapia as a result of the corrective treatment with a sufficient concentration of this substance.

MATERIALS AND METHODS

150 individuals of tilapia, total length (TL) 8 ± 1 cm; weight (W) 12 ± 2 g, infested with *Cichlidogyrus* spp. in aquaculture habitual conditions [Prevalence (PREV) and Mean Abundance (ABUND)] (Margolis *et al*., 1982): 53.33%; 0.96 ± 1.25 parasites per fish, respectively], were obtained from Center of Investigation and Advanced Studies of National Polytechnique Institute, Mérida Unit (CINVESTAV-Mérida) facilities. Fish were acclimated for 15 days at static concrete tanks of 1.2 m^3 with independent aeration by continuous bubbling in acclimatized rooms. Fish were maintained in a 14 h light: 10 h darkness photoperiod and they were fed twice a day with commercial pellets (Tilapia Chow, 40% protein, 4% fat, 5% fiber; the size of pellet 1.5 mm) *ad libitum* until three days before the experiment. The remaining food pellets and fecal matter accumulated at the bottom of the acclimation tanks were removed daily to ensure a proper hygienic condition. After the acclimation period, individuals were randomly distributed in 15 glass aquariums of 120 L each one (10 fish per aquarium) with independent aeration for each experimental unit by continuous bubbling.

Based on previous data about the effective concentration of formalin against *Cichlidogyrus* spp. infecting tilapia (Vidal-Martínez *et al*., 2002; Sandoval-Gío *et al*., 2008), the therapeutic bath consisted of four treatments with concentrations of 0.5, 1.0, 1.5 and 2.0 mL of formalin (Sigma Aldrich, México, 37% dilution) per liter of water, respectively, and one negative control free of the substance, with three repetitions each one. The corrective treatment was done in same aquariums post acclimation by individually adding the substance on each one, to avoid stress by handling. The test lasted 40 min and after that fishes were maintained in the quarantine area for later parasitological analysis.

A digital oximeter (YSI 550) was used to measure once a day the water dissolved oxygen and temperature. Also, the water samples were collected to weekly analyze other water quality variables (pH, nitrites, and ammonium) at the Marine Chemistry Laboratory of CINVESTAV-Mérida (Eaton *et al*., 1998). Fish were not fed throughout the bioassay to avoid physical and chemical changes in water.

The effective therapeutic concentration (ETC) was measured as the formalin percentage of effectiveness against prevalence and abundance for every concentration, and it was calculated using the subtraction of PREV and ABUND in each concentration and values of the control group (Fajer-Ávila *et al*., 2007).

For stress and immune response determination, two groups were compared: 30 tilapias (TL: 8 ± 1 cm; W: 12 ± 2 g) without *Cichlidogyrus* infestation were maintained in CINVESTAV-Mérida quarantine area for three months before bleeding (CTRL Group). These

fishes were obtained of a free of pathogens experimental culture by donation of University of Stirling (Scotland, UK) (Sandoval-Gío *et al*., 2013). On the other hand, 30 fishes (TL: 9 ± 1 cm; W: 12 ± 2 g), obtained from CINVESTAV facilities were treated with ETC of formalin against *Cichlidogyrus* obtained previously (FORM Group). Posterior to treatment, tilapias of FORM Group were carefully held and introduced for recuperation, in fiberglass with fresh and clean water, for 24 h. Fishes were not supplied with food during this period. After that, fishes were maintained in CINVESTAV-Mérida facilities quarantine area for three months (Sandoval-Gío *et al*., 2008).

The serological samples were obtained from 15 fishes of each group (FORM and CTRL), randomly bled four times as follows: Time 0 (T0): tilapias were bled one day before formalin bath; Time 1 (T1): fishes were bled one hour after formalin treatment; Time 2 (T2): fish were bled 3 days after formalin treatment; Time 3 (T3): tilapias were bled 3 months after formalin treatment. Blood samples were taken, without anesthesia, from the caudal vein, into heparinized and disposable syringes, with a 22-G needle. This study was conducted following institutional guidelines for the protection of animal welfare.

Before coagulation, one drop of blood of each sample was placed in a clean microscope slide to make blood smears. For differential counting, blood smears were fixed in methanol for 3 min, stained with Giemsa stain (Sandoz, 1973), and were observed by triplicate under a light microscope (VELAB, México) at 100x. The diagnostic criterion was to count 100 leukocytes and classified following Hrubec *et al*. (2000).

The remaining blood was left to coagulate for 30 min into Eppendorf tubes at room temperature (RT), and centrifuged at 1,500 *g*, for 10 min, and the serum was transferred into new tubes and stored at -80°C until posterior analysis.

The primary response to stress in tilapia was evaluated using a cortisol bioassay using 96 wells microplate according to Cortisol, Express EIA Kit (Cayman Chemical Company, USA). Cortisol values were recorded in an ELISA spectrophotometer at 405 nm (Dynex Technologies Ltd., MRX 20100, Worthing, UK). Glucose was analyzed with a SPIN REACT kit (Girona, Spain) according to the oxidase-peroxidase colorimetric method (Trinder, 1969), and adapted to 96 wells microplate (Sandoval-Gío *et al*., 2013). Absorbance values were measured in an ELISA reader at 550 nm (Dynex Technologies Ltd., MRX 20100, Worthing, UK). Lysozyme activity was measured employing a turbidimetric assay microplate (Parry *et al*., 1965; Lee & Yang, 2002). In each well, a 250 µL suspension of lyophilized *Micrococcus lysodeikticus* (0.2 mg mL^{-1} in 0.04 M of phosphate buffer solution NaCl; pH 5.75) and 10 µL of *O. niloticus* serum were placed, by triplicate. Egg lysozyme was used as a standard (SIGMA, USA). The reduction in absorbance was recorded from 1 to 11 min at 22°C, in an ELISA reader (Dynex, MRX, Worthing, UK). One unit of lysozyme activity (U mL^{-1}) was defined as a decrease in absorbance (0.001 min L^{-1}).

Data from all experiments were checked for normality and homogeneity of variance by both Wilk-Shapiro analysis and χ^2 Bartlett. When they did not achieve normality, a natural logarithm (ln+1) was used for transformations. Differences from control values were compared statistically through a One-Way ANOVA and Tukey test at $P < 0.05$ for rejection level. All statistics were carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., USA).

RESULTS

During the experiment, no changes were observed in the water physical and chemical variables (mean ± SD: temperature = 24 ± 3ºC, dissolved oxygen = 5.8 ± 0.5 mg L^{-1}, pH = 7.98 ± 0.45, nitrite = 2.91 ± 1.01 mg L^{-1}, ammonium = 0.09 ± 0.03 mg L^{-1}); nevertheless, at the end of trial, a slow decrease in dissolved oxygen concentration was detected without significance.

Throughout formalin exposure, no mortality in fishes of FORM Group treated with ETC was observed. However, signs of over toxicity were observed at 2.0 mL L^{-1}, *e.g.*, erratic movements and low stability (Herwig, 1979). Prevalence (P%) and abundance (A) were 100% at a concentration of 1.5 and 2.0 mL L^{-1}. P% and A for 1.0 mL L^{-1} doses were 93.75 and 96.87, respectively. Thus, effective therapeutic concentration (ETC) of formalin was established at 1.5 mL L^{-1} for 40 min. After the bioassay, the effectiveness percentages against prevalence (P%), and effectiveness against abundance (A) for each treatment can be observed in Table 1.

For cortisol determination, for CTRL Group, mean values ranged from 37 to 47 ng mL^{-1} without significant differences for all bleeding times ($P < 0.05$) (Fig. 1). For FORM Group, cortisol value at T1 (~326 ng mL^{-1}) was significantly higher than CTRL Group ($P < 0.05$) (Fig. 1).

For glucose determination, in FORM Group there were significant differences in T1 (~119 mg dL^{-1}) (1 h after treatment) concerning T1 (~59 mg dL^{-1}) of CTRL Group ($P > 0.05$). Also, the increase of glucose in FORM Group (T1) was without significance when compared with T2, but this peak was statistically higher than T0 and T3 of FORM Group ($P > 0.05$) (Fig. 2). In

Table 1. Mean values of prevalence, and mean abundance of *Cichlidogyrus* spp. in *Oreochromis niloticus* for different concentrations of formalin after of the bioassay of effectiveness against prevalence (P%) and abundance A. Prevalence and abundance before treatment were 53.33 and 0.96 ± 1.25, respectively. SD: standard deviation.

Concentration (mL L^{-1})	Prevalence (after)	Abundance (after) ± SD	P%	A
2.0	0	0	100	100
1.5	0	0	100	100
1.0	3.33	0.03 ± 0.56	93.75	96.87
0.5	33.3	0.26 ± 0.46	37.55	72.91
0.0	78.1	0.99 ± 0.84	-----	-----

the CTRL Group, there were no significant differences in values among bleeding times ($P > 0.05$) (Fig. 2).

The results of all lysozyme concentration values indicated no statistical differences between the FORM and CTRL Groups (ranged from 204 to 232 UI mL^{-1}) ($P > 0.05$) (data not shown).

Differential counting exhibited four types of white cells: lymphocytes, thrombocytes, granulocytes and monocytes. For CTRL and FORM groups, lymphocytes were the cell line most abundant followed by thrombocytes (Table 2). Granulocytes and monocytes were registered as less abundant in the differential counting. There were no significant differences in the assembled differential counting of several leucocytes types among studied groups ($P > 0.05$).

DISCUSSION

Formalin is one of the chemical pollutants most used in aquaculture procedures. This substance is the active principle of three products that of US has approved to therapeutic use against several ectopara-sites in fish farming for the last two decades (Holladay *et al*., 2010; Nasser *et al*., 2017).

Up to date, in Mexico, even with the robust economic importance of tilapia in aquaculture, there is scarce information about the appropriate doses of formalin required to eradicate monogeneans in *Oreochromis niloticus*. For example, Mexican aquaculture authorities recommend a bath of 0.25 mL L^{-1} of this chemical for 1 h to control monogeneans in fish (SENASICA, 2008). This protocol is described for monogeneans in general despite the importance of C*ichlidogyrus* spp. as the culprit chief of monogenean pathogenesis, almost all sanitary protocols have been developed to control *Dactilogyrus* spp. (Salgado- Maldonado & Rubio-Godoy, 2014; Rindoria *et al*., 2015). In this sense, in Yucatan tilapia farming,

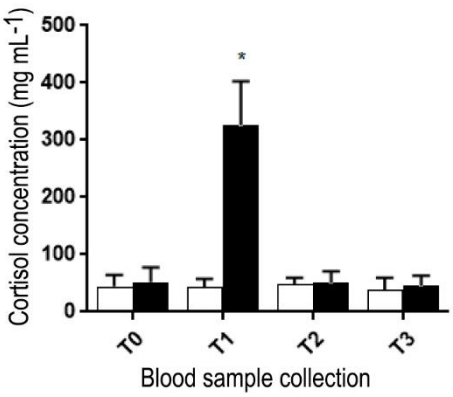

Figure 1. Cortisol concentration (mean ± SD) (n = 15) in *Oreochromis niloticus* exposed to 1.5 mL L^{-1} of formalin for 40 min (ETC) at four blood sample times. White bars: CTRL Group; black bars: FORM Group. *Different from control ($P < 0.05$).

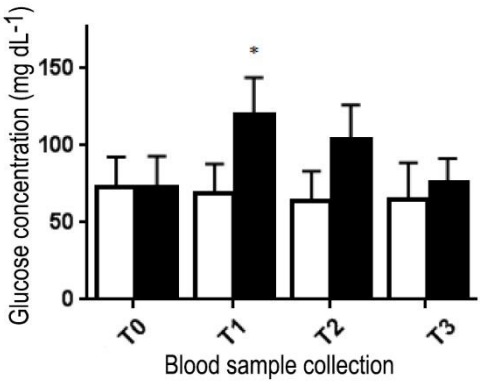

Figure 2. Glucose concentration (mean ± SD) in *Oreochromis niloticus* exposed to effective concentration therapeutic at four blood sample times. White bars: CTRL Group; black bars: FORM Group. *Different from control ($P < 0.05$).

C. sclerosus was found as the most prevalent monogenean species (Paredes-Trujillo *et al*., 2016).

In this research, the effective therapeutic concentration (ETC) of formalin found to control cichlidogyriasis in tilapia was of 1.5 mL L^{-1} for 40 min, with a 100% of effectiveness against prevalence and abundance of this parasite, establishing an optimal margin of use with high sensitivity. In another study, the effective therapeutic dose recorded was of 2 mL L^{-1} for 40 min to eradicate *Cichlidogyrus* sp. in *O. niloticus* (Sandoval-Gío *et al*., 2008). Differences between these data may be substantially related to the species development stage, because the fish in this study were juvenile, in contrast with adult organisms analyzed by Sandoval-Gío *et al*. (2008).

Table 2. Differential counting (in percentage, mean ±SD) of organic defense cells in *Oreochromis niloticus* under formalin treatment (Experiment 2). CTRL: Control Group, fishes without treatment, FORM: Formalin Group, fish under formalin treatment ($P > 0.05$). SD: standard deviation.

Cell type leukocyte	CTRL (n = 60)	FORM (n = 78)
Lymphocytes	76 ± 4	74 ± 5
Thrombocytes	21 ± 4	20 ± 4
Granulocytes	2 ± 1	4 ± 2
Monocytes	1 ± 1	2 ± 2

Although there is evidence to suggest adverse physiological effects caused by several substances as origin of stress and depleting immunity for fish (Williams & Wooten, 1981; Ellis, 1989; Girón-Pérez *et al*., 2009); the information about the antagonistic effects of formalin on immune physiology of tilapia when utilized at therapeutic levels is inconsistent (Holladay *et al*., 2010).

Our results evidence an increase of cortisol levels (326 ng mL^{-1}) and glucose (~119 mg dL^{-1}) 1 h after the end of the formalin treatment, which returned at basal levels at the last two bleeding times. Several authors have demonstrated the kinetic metabolism of the stress, concurring in the first liberation of glucocorticoids hormones, especially cortisol, from the interrenal tissue of fish, in response to tension agent, which represents a primary response to the stress (Barton 2002; Cnaani *et al*., 2004). After that, a consequent production of serum glucose occurs to provide energy to the organism and in consequence, to confront the stressful event, representing this mecha-nism a secondary response to the stress (Sanches *et al*., 2015).

Values from 100 to 200 ng mL^{-1} of cortisol have been observed in tilapias exposed to severe stress (Foo & Lam, 1993; Morgan *et al*., 1997). Hoseini & Tarkhani (2013), found concentrations of cortisol up to 20 times above the basal levels in *Carassius auratus* exposed to 0.25 mL of formalin for 30 min. This value was maintained other 30 min after finishing the experimental treatment. These authors also found a decrease in cortisol levels after 3 and 24 h suggesting the fish recovery from the chemical exposure, though to levels significantly higher than registered at the beginning of the treatment.

Levels of cortisol in fish depend on several factors as the severity of the stressor agent, its duration, and its exposition, as well as the health of the organisms (Foo & Lam, 1993; Gesto *et al*., 2015). It has also been established that after exposition to stress situations, the removal of this condition results in a rapid return to the basal hormonal level, this situation was similarly observed in our experiment (Guerriero & Ciarcia, 2006; Tahir *et al*., 2018).

Barton *et al*. (1987) postulated that the attenuation of the interrenal response due to the lack of the stressful influence could be caused by a reduction of the hormone ACTH secretion, or by the hypothalamic secretion of the liberating factor of the corticotrophin decrease, resulting in a decline of the plasma cortisol. Thus, the reestablishment of cortisol level in this study may be associated with an acceptable interval of less than one hour of treatment concurring with the ETC provided.

For the evaluation of serum glucose, at the beginning of our experiment, the fish under treatment had similar values with controls (~72 mg dL^{-1}). Later, in FORM Group, serum glucose increased (~119 mg dL^{-1}) and it remained increased three days after finished the bath with formalin, registering stabilization only three months later (T3).

Other researchers have found increased values of glucose after experimental treatment with formalin in fish, nevertheless, and like the cortisol tendency, some hours post-treatment the glucose values returned to basal levels (Kakuta *et al*., 1991; Araújo *et al*., 2004).

It is possible that in our experiment, the cortisol concentration peak caused an associated increase in the serum glucose and, after that, extreme reduction of cortisol to basal levels at T2, originating the slow decrease of serum glucose registered after T1.

Concerning the lysozyme, this is a molecule that is activated in innate defense processes of most vertebrates, and its quantification is taking relevancy in aquaculture settings (Taoka *et al*., 2006; Badr *et al*., 2014). The influence of the stress on the activity of lysozyme remains imprecise since several authors have notified contradictory information concerning its variation in response to stressors agents (Domínguez *et al*., 2005). In our work, no significant differences were found in values of lysozyme either at FORM or CTRL group. Although Caruso & Lazard (1999), described significant declines of lysozyme in tilapias under chronic stress in comparison with control groups. Moreover, Demers & Bayne (1997) observed equivalent increment concentrations of both cortisol and lysozyme, after acute stress exposition. It is not understood why in our tilapias the mechanisms of enzyme activation have not liberated in favorable form as a response of concomitant high levels of cortisol, after acute stress (Môck & Peters, 1990; Bulut *et al*., 2012). One explanation according to the lack of activation of lysozyme activity could be associated with the strong correlation of this enzyme to the phagocytic activity. In the differential counting of leukocytes, the mean number of granulocytes and monocytes cells

involved in inflammatory and phagocytic processes had an observable increase, but not significantly, in fishes under formalin treatment. These results are concurrent with observations that formalin produces a process of inflammation in exposed tissues, especially in gills, due to respiratory insufficiency that generates in fishes (Prieto, 1987). Silveira-Coffigny *et al*. (2004) coincidently found increasing granulocytes levels due to an induced infection of *Corynebacterium* spp. and overdose of green malachite in *Oreochromis aureus*. In contrast, slow lymphocytopenia observed in our tilapias could be correlated with a compensatory intercellular regulation (Pickering, 1998).

In conclusion, 1.5 mL L^{-1} of formalin for 40 min resulted in 100% of effectiveness against prevalence and abundance of *Cichlidogyrus* spp. in tilapia. The results of this research regarding the values of effective concentration of formalin in tilapia are relevant to commercial use in aquaculture facilities of Yucatan Peninsula, especially in the near future with the likely increase of these activities.

Peaks of cortisol and glucose were observed in tilapias of FORM Group at one hour after the end of the treatment with formalin, but these values returned to their basal levels at the end of this study. It is possible that short-time tests for evaluation of lysozyme are not representative to assess the state of the animal exposed to a chemical agent.

The regulatory preservation of stress and immunity indicators obtained here could be a signal that ensures the fish recovery and supports the use of formalin if constant monitoring of physicochemical parameters of water and care of other aquaculture proceedings are taken. For example, a decrement, without significance, in values of dissolved oxygen was detected at the end of our bioassay, according to the evidence that high concentrations of formalin origins high saturation of water. Thus, it is recommendable to monitor physical and chemical parameters in fishes under treatment to avoid an additional increase in stress according to the evidence of formalin origins high saturation of water.

This study provides evidence of 100% of the effectiveness of corrective therapeutic formalin baths at a concentration of 1.5-2.0 mL L^{-1} without compromising the effector cells and enhancing stress. This procedure was performed in laboratory settings. An extension of this study has been expanded to farms to assess its effectiveness in semi-intensive tilapia culture.

ACKNOWLEDGMENTS

To the National Council of Science and Technology of Mexico (CONACYT), for the Ph.D. scholarship to JJSG (Nº 209205/129336).

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Received: 5 April 2018; Accepted: 17 July 2018