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*Research Articles*

# Homeopathy improves production and hatching probability of zebrafish eggs

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**ABSTRACT.** The homeopathy has been practiced for many years, mainly using rodent as a research model. Zebrafish have been used as an animal model in different scientific areas and may represent an important model in studies with this therapy. This paper aimed to evaluate the action of Homeopatila 100® on the growth, the action on the liver and reproduction of zebrafish. The study was carried out with 192 six months old animals. The experimental period was three months, being evaluated the growth, the hepatosomatic and viscerosomatic indexes, the number of hepatocytes per area and the percentage of glycogen in these cells, performing the hepatic histology of males and females. It was evaluated the reproduction of the animals, checking the production and probability of hatching of the eggs. It was found that homeopathy increases the number of hepatocytes and glycogen in females, as well as improved the reproductive indices evaluated.

**Keywords:** *Danio rerio*; zebrafish; glycogen; hepatocytes; liver; reproduction

## INTRODUCTION

Homeopathy, based on the principle of the "Law of Similars" and the use of drugs that retain biological activity, after dilutions and succussions (Ernst, 2002), when applied collectively is considered as population homeopathy. This form of administration considers a group of individuals kept under the same environmental conditions as a single organism and is widely used in the treatment of production animals (Benez *et al*., 2004). Homeopathic products have some advantages compared to other therapeutic forms, for example, they are easy to administer, do not leave residues in the body and do not contaminate the environment, which is characterized as important factors in food production (Benez *et al*., 2004).

The effect of homeopathy on productive performance and meat quality in fish has been evaluated, mainly for Nile tilapia. Remarkable results have been observed, such as increased muscle hypertrophy, survival, decrease in lipid content and omega 6:3 ratio (Júnior *et al*., 2012; Andretto *et al*., 2014). Scientific studies report that homeopathy causes minor liver damage, greater storage of glycogen and a lower hepatosomatic rate, besides not influencing the quality of meat and derived products and improving breeding rates, such as spawning frequency and larvae hatching (Valentim-Zabott *et al*., 2008; Siena *et al*., 2010; Braccini *et al*., 2013; Andretto *et al*., 2015; Fuzinatto *et al*., 2015; Lima *et al*., 2015; Pinheiro *et al*., 2015) demonstrating that the product Homeopatila 100®, when acting as a hepatoprotective, can influence important factors in the production, growth, reproduction, and quality of meat.

In addition to the production of animals, homeopathy is studied in experimental models such as rats, mice and non-human primates (Bonamin *et al*., 2015; Chatfield *et al*., 2016). In this group, rats and mice correspond to 72% of the research of this therapy in the last 10 years, with the main purpose of using them as models of disease (Chatfield *et al*., 2016). In addition to rats and mice, the *Danio rerio* fish popularly known as zebrafish has stood out as an animal model.

The use of zebrafish is due to its genetic characteristics, which have a fully sequenced genome, where it is found that 71% human genes have at least one orthologous gene in zebrafish (Vilella *et al*., 2008). Moreover, 81% of the human genes related to the cause of diseases are orthologs of the genes of this fish (Howe *et al*., 2013). Besides the genetic factors, some characte-

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characteristics favor its use as an animal model in the laboratory, such as small size, robustness, short life cycle, generation of large numbers of offspring and mainly ease and low cost to keep it in research environments (Nasiadka & Clark, 2012). Zebrafish can also be used in model studies in comparison to other animals, especially other fish species, besides being considered a standard organism concerning humans. The use of zebrafish as an animal model and a research tool in several areas in studies related to homeopathy is emphasized.

The evaluation of the effect of the homeopathic complex Homeopatila 100® can elucidate the results already obtained in Nile tilapia, as well as to provide more information that allows its use in other aquatic organisms. This study aimed to evaluate the effect of the homeopathic complex Homeopatila 100® on the productive performance, reproduction and evaluation of the liver of zebrafish.

## MATERIALS AND METHODS

### Ethics committee

The manipulations and experiments with the animals were carried out following the regulations established by the Ethics Committee of the State University of Maringá (SUM), with the approval of the project with protocol number: 4045281116.

### Animals and experimental period

The study was developed at the Zebrafish Laboratory of SUM, from January to April 2017, totaling 90 days. Six-month-old zebrafish with 0.30 ± 0.08 g in weight and 28.30 ± 2.06 mm in total length were kept in 30 L aquaria, with constant aeration and 14:10 h light-dark cycle, as recommended for the species.

### Design and experimental diets

The study was carried out in a completely randomized design, with four treatments and four replications. A total of 192 fish were used, distributed in 16 aquaria, totaling 12 animals per experimental unit. Fish were fed four times a day (08:00, 11:00, 14:00 and 17:00 h) to apparent satiety, with Bern Aqua commercial diet, containing 57% CP and 300-500 μm particle size, with the inclusion of the homeopathic complex Homeopatila 100® (RealH). The treatments consisted of inclusion levels of the homeopathic complex, being a control treatment (20 mL 30º gl Hydroalcoholic solution) and three treatments with the inclusion of 20, 40 and 60 mL $kg^{-1}$ of Homeopatila 100® in the feed (Table 1).

### Experimental methodology

During the experimental period, initial and final biometry was performed, and the length (mm) and weight (g) of all the animals used in the study were measured. Fish were fasted for 24 h and then anesthetized with a solution containing water and 100 ppm clove oil (Eugenol) as described by Grush *et al.* (2004), weighed and measured.

**Table 1.** The composition of the homeopathic nucleus Homeopatila 100®. REALH (www.realh.com.br).

| Compound | 1000 $g^{-1}$ |
|---|---|
| Iodum | $10^{-24}$ |
| Sulfur | $10^{-60}$ |
| Natrum muriaticum | $10^{-400}$ |
| Streptococcinum | $10^{-60}$ |
| Vehicle (30º gl ethyl alcohol) | Q.s.p. |

Ninety days after the onset of the experiment, the final biometry was performed to evaluate the productive performance. Six fish from each experimental unit (three males and three females) were euthanized by immersion in ice, destined for histological assessment of the liver and obtaining the hepatosomatic and gonadosomatic indices. Three couples per experimental unit were assigned to reproduction and evaluation of reproductive parameters.

### Productive performance

With the final biometric data, animal performance was obtained according to the sexes of the parameters total weight (g); total length (mm); liver weight (g); and gonad weight (g). In addition, the hepatosomatic index (HI) = (liver weight / fish weight) × 100; and gonadosomatic index (GI) = (gonad weight / fish weight) × 100 were calculated.

### Histology

Histological evaluation was conducted in the liver of four animals per experimental unit, two males and two females. The samples were collected, washed with a physiological solution (0.9% NaCl) fixed in Bouin for 6 h and later stored in 70% alcohol. The material was dehydrated in an ascending series of alcohol, diaphanized in xylol and embedded in paraffin to obtain the histological sections in the Laboratory of animal histology, Department of Morphology, State University of Maringá.

For hepatocyte count per area, the slides were stained with Hematoxylin and Eosin, while for verification of intracellular glycogen (%), we used the staining with periodic acid Schiff (PAS) + hematoxylin. The slides were photographed under Olympus optical microscope at 40x magnification for hepatocyte counts and 100x for glycogen verification. Subsequent analyses were performed using Image Pro Plus software.

## Breeding and reproductive parameters

At the end of 90 experimental days, three couples of each replicate were selected for mating. They were removed from the aquarium and housed in 1.5 L reproduction structures, composed of an external structure and an internal structure with a screen bottom so that after spawning, the eggs remained at the bottom of the external structure, protected from predation.

In the case of spawning, eggs were collected, sanitized with water and kept in Petri dishes, in the amount of 50 eggs per dish. The number of eggs per female and the hatching probability (%) = (number of hatched larvae/total number of eggs) × 100 were analyzed.

## Water quality

The water temperature was verified daily, in the morning (08:00) and the afternoon (17:00), with the aid of a digital thermometer, remaining with an average of 27.2 ± 1.4ºC during the experimental period. Values of pH and dissolved oxygen (DO) were measured weekly using a YSI multiparameter probe, maintaining the values of 7.3 ± 0.8 and 6.5 ± 0.6 mg $L^{-1}$ for pH and DO, respectively.

## Statistics

The data obtained were subjected to Bayesian analysis. For this, the means were tested for normal distribution with homogeneous variance ($Y|\mu, \sigma^2 \sim \text{Normal}(\mu, \sigma^2)$, $\mu$ real, $\sigma^2 = \sigma^2$ e $> 0$); normal distribution with heterogeneous variance ($Y|\mu, \sigma^2 \sim \text{Normal}(\mu, \sigma^2)$, $\mu$ real, $\sigma^2 \neq \sigma^2$ e $> 0$) and gamma distribution ($Y|\alpha, \beta \sim \text{Gama}(\alpha, \beta), \alpha \text{ e } \beta > 0$, whose mean is $\alpha/\beta$). The most parsimonious distribution was defined following the Deviance Information Criterion (DIC) (Spiegelhalter *et al.*, 2002). Due to the characteristics of the parameters, survival and hatching followed binomial distribution ($Y|\theta \sim \text{Binomial}(n, \theta)$, that is, the observed value in mortality and non-hatched larvae, with probability of survival and hatching; number of eggs and hatched larvae, Poisson-gamma distribution ($\gimel|Y \sim \text{Gama}(\alpha + t, \beta + n)$, whose ($\gimel$) is the mean number of eggs and hatched larvae.

Non-informative a priori distributions were considered for all parameters under study. Obtaining the marginal distributions a posteriori for the parameters were obtained using the BRugs package of R (R Development Core Team, 2018) 1,000,000 values were generated in an MCMC (Monte Carlo Markov Chain) process, considering a sampling period of 100,000 initial values and withdrawal interval of 10. The convergence of the chains was verified by the criteria of Heildelberger & Welch (1983) and Geweke (1992) and, implemented in the *coda* package of R. The significance of the treatments was checked by the presence or not of zero in the respective 95% credible interval (ICr (Δ, 95%) for the contrasts.

# RESULTS

Differences were detected in the productive parameters of total weight and hepatosomatic index for males and females, respectively (Table 2). The highest final weight for males was verified for the control treatment, without the inclusion of the homeopathic complex and with 20 mL inclusion per kg feed, while the animals subjected to treatments with 40 and 60 mL of the product had lower weight, differing only with control. About the hepatosomatic index, the females fed a diet containing 20 mL Homeopatila 100® presented lower value when compared to the females of the control treatment.

Higher values were observed for the females in all parameters evaluated, mainly for the hepatosomatic and gonadosomatic indices. The treatments did not influence the survival of the animals, which was above 90% in all levels of Homeopatila 100® evaluated (0 mL $kg^{-1}$: 93.99%, 20 mL $kg^{-1}$: 90.02%, 40 mL $kg^{-1}$: 94.00%, and 60 mL $kg^{-1}$: 91.99%).

There was a difference in the number of hepatocytes and the percentage of glycogen (Table 3). The hepatocyte count varied with the treatments considering both sexes, a lower number was observed for the control treatment. For the females, it was observed that the animals fed diets containing 20 and 40 mL of the homeopathic complex per kg of diet had a higher number of hepatocytes; glycogen occupied from 31.47 to 49.31% of the total hepatocyte area, being lower in the control treatment. In females fed a diet containing 40 mL of the product per kg of diet, it occupied 49.31% of the total area of the hepatocytes, differing from the other treatments. The reproductive parameters also differed, with higher values for the treatments with 40 and 60 mL $kg^{-1}$ for the number of eggs, being 416 and 402, respectively (Fig. 1).

There was a higher hatching probability, with a higher number of larvae for the treatment with 60 mL of Homeopatila 100® per kg of feed and a lower value for the control treatment (Fig. 2).

# DISCUSSION

There were higher values of the productive parameters for the females compared to the males. At the end of the experimental period, the animals used were nine months old and were able to reproduce, since zebrafish

**Table 2.** Productivity parameters of zebrafish *Danio rerio* fed different levels of Homeopatila100® (Mean, followed by the range in parentheses and standard deviation (SD)). Different superscript letters in the same column indicate differences between treatments.

| Variables | Treatments (mL kg$^{-1}$) | Males | | Females | |
|---|---|---|---|---|---|
| | | *A posteriori* means | SD | *A posteriori* means | SD |
| Total weight (g) | 0 | 0.76$^{a}$ (0.71-0.83) | ±0.072 | 1.09$^{a}$ (0.95-1.23) | ±0.07 |
| | 20 | 0.74$^{ab}$ (0.68-0.80) | ±0.052 | 1.16$^{a}$ (1.02-1.29) | ±0.07 |
| | 40 | 0.67$^{b}$ (0.6182-0.7371) | ±0.033 | 1.11$^{a}$ (0.98-1.25) | ±0.07 |
| | 60 | 0.67$^{b}$ (0.61-0.73) | ±0.033 | 1.02$^{a}$ (0.88-1.15) | ±0.07 |
| Total lenght (mm) | 0 | 40.88$^{a}$ (39.13-42.64) | ±0.884 | 43.92$^{a}$ (42.28-45.58) | ±0.83 |
| | 20 | 39.51$^{a}$ (37.76-41.28) | ±0.887 | 43.83$^{a}$ (42.18-45.50) | ±0.83 |
| | 40 | 40.52$^{a}$ (38.77-42.29) | ±0.886 | 44.05$^{a}$ (42.40-45.71) | ±0.83 |
| | 60 | 40.11$^{a}$ (38.36-41.85) | ±0.881 | 43.74$^{a}$ (42.10-45.38) | ±0.83 |
| Liver weight (g) | 0 | 0.008$^{a}$ (0.005-0.012) | ±0.001891 | 0.042$^{a}$ (0.030-0.059) | ±0.009 |
| | 20 | 0.008$^{a}$ (0.006-0.012) | ±0.001614 | 0.032$^{a}$ (0.026-0.040) | ±0.004 |
| | 40 | 0.006$^{a}$ (0.003-0.010) | ±0.001710 | 0.033$^{a}$ (0.026-0.042) | ±0.009 |
| | 60 | 0.008$^{a}$ (0.005-0.014) | ±0.002337 | 0.035$^{a}$ (0.028-0.045) | ±0.005 |
| Gonad weight (g) | 0 | 0.008$^{a}$ (0.006-0.012) | ±0.001925 | 0.107$^{a}$ (0.077-0.150) | ±0.025 |
| | 20 | 0.010$^{a}$ (0.007-0.014) | ±0.001623 | 0.194$^{a}$ (0.111-0.343) | ±0.107 |
| | 40 | 0.006$^{a}$ (0.004-0.013) | ±0.002600 | 0.145$^{a}$ (0.121-0.173) | ±0.024 |
| | 60 | 0.008$^{a}$ (0.006-0.009) | ±0.000892 | 0.140$^{a}$ (0.089-0.139) | ±0.066 |
| Hepatosomatic index (%) | 0 | 1.05$^{a}$ (0.753-1.472) | ±0.1983 | 4.35$^{a}$ (3.40-5.58) | ±0.583 |
| | 20 | 1.13$^{a}$ (0.762-1.699) | ±0.2654 | 2.770$^{b}$ (2.12-3.64) | ±0.411 |
| | 40 | 1.02$^{a}$ (0.517-1.630) | ±0.3835 | 3.103$^{ab}$ (2.32-3.04) | ±1.489 |
| | 60 | 1.26$^{a}$ (0.855-1.875) | ±0.2984 | 3.500$^{ab}$ (2.70-3.45) | ±0.526 |
| Gonadosomatic index (%) | 0 | 1.070$^{a}$ (0.758-1.612) | ±0.3056 | 11.010$^{a}$ (8.95-13.67) | ±1.269 |
| | 20 | 1.287$^{a}$ (0.881-2.027) | ±0.4008 | 15.030$^{a}$ (10.18-23.00) | ±4.835 |
| | 40 | 1.040$^{a}$ (0.643-2.044) | ±0.9786 | 13.130$^{a}$ (11.61-14.88) | ±9.355 |
| | 60 | 1.104$^{A}$ (0.881-1.421) | ±0.1382 | 12.780$^{a}$ (9.54-17.25) | ±2.574 |

**Table 3.** Hepatic parameters of zebrafish *Danio rerio* fed different levels of Homeopatila 100® (mean, followed by the range in parentheses and standard deviation (SD)). Different superscript letters in the same column indicate differences between treatments.

| Variables | Treatments (mL) | Males | | Females | |
|---|---|---|---|---|---|
| | | *A posteriori* means | SD | *A posteriori* means | SD |
| Number of hepatocytes | 0 | 251$^{b}$ (238-264) | ±6 | 302$^{c}$ (287-318) | ±8 |
| | 20 | 293$^{a}$ (279-307) | ±7 | 441$^{b}$ (425-458) | ±9 |
| | 40 | 286$^{a}$ (273-300) | ±7 | 432$^{b}$ (416-449) | ±8 |
| | 60 | 284$^{a}$ (270-297) | ±7 | 406$^{a}$ (388-423) | ±9 |
| Glycogen (%) | 0 | 34.24$^{a}$ (31.71-36.97) | ±1.33 | 31.47$^{c}$ (29.50-33.56) | ±1.03 |
| | 20 | 33.38$^{a}$ (31.41-35.47) | ±1.03 | 35.77$^{b}$ (32.51-39.36) | ±1.74 |
| | 40 | 35.91$^{a}$ (33.80-38.13) | ±1.09 | 49.31$^{a}$ (47.46-51.25) | ±0.96 |
| | 60 | 35.75$^{a}$ (33.57-38.05) | ±1.14 | 36.62$^{b}$ (34.55-38.79) | ±1.08 |

specimens may show sexual maturity within 90 days (Spence *et al*., 2007). Because they were kept under ideal conditions of environment and management, development was favored with increasing gonadal weight.

The ovary exerts influence on the body weight of females, due to the occupation of a large part of the celomatic cavity, demonstrated by the gonadosomatic index. The results found for the gonadosomatic index were within the values described for zebrafish by Chang *et al.* (2013) and Gonzales & Law (2013) in their control treatments, with values close to 1% for males and 13% for females.

The liver of zebrafish is composed of three lobes, two lateral and one ventral (Goessling & Sadler, 2015). It is an important organ of the organism that exerts different functions, among them, those related to metabolic activities (synthesis of proteins, lipids, carbohydrates and vitellogenin) and in responses immunological. The liver is composed of hepatocytes,

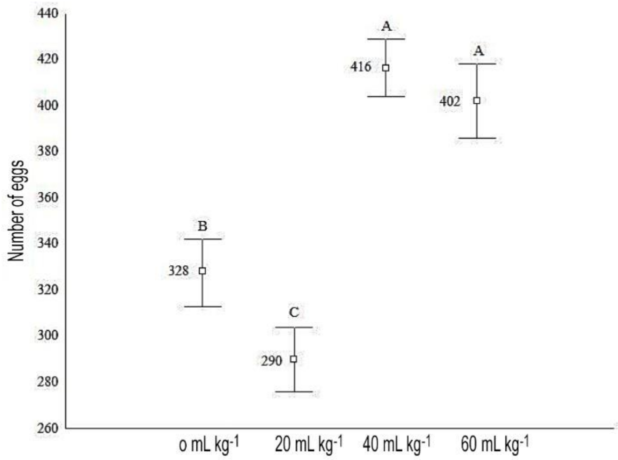


**Figure 1.** The mean number of eggs per zebrafish *Danio rerio* female fed different levels of Homeopatila 100®. Different letters indicate statistically significant differences between treatments.

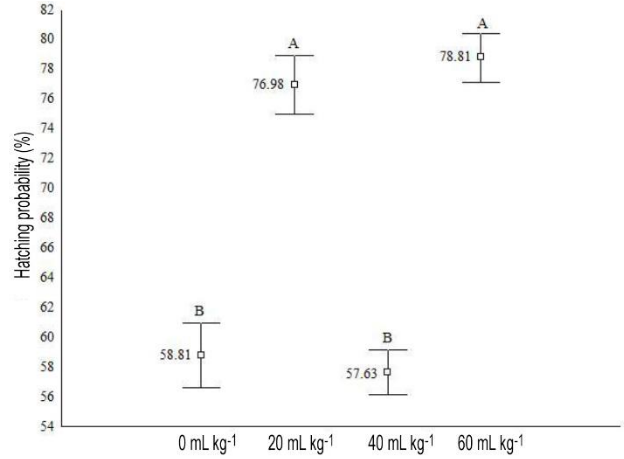


**Figure 2.** Hatching probability of zebrafish *Danio rerio* larvae fed different levels of Homeopatila 100®. Different letters indicate statistically significant differences between treatments.

which are the primary functional cells and where reserve substances such as glycogen and lipids are accumulated.

The amount of these substances interferes with the liver weight and consequently in the hepatosomatic index. However, in the present study, there was no change in the weight of this organ about the treatments, for both males and females. There was variation in the index for females fed diets containing 20 mL Homeopatila 100® per kg of feed when compared to the control. This result may be due to their weight, which, although not statistically different, was higher than that observed for the other treatments.

This result contrasts with Pinheiro *et al.* (2015), who studied the same levels of Homeopatila 100® in tambaqui (*Colossoma macropomum*), and found a higher hepatosomatic index for this treatment when compared to the inclusion of 40 mL per kg of diet. The hepatosomatic index from 1.01 to 1.3% for males is similar to that described by Chang *et al.* (2013) for zebrafish, being 1.59% for individuals of the same sex. On the other hand, it is higher considering the females, which was 2.81% and in the present study, the values observed ranged from 2.70 to 4.34%.

There was variation regarding the number of hepatocytes, with higher values in the animals treated with the homeopathic complex Homeopatila 100® in comparison to the control. For females, a higher number of these cells were verified, mainly with diets containing 20 and 40 mL per kg of this product in the diet. These results corroborate with Braccini *et al.* (2013), where a higher number of hepatocytes was verified in the liver of Nile tilapia fed Homeopatila 100® (40 mL $kg^{-1}$) compared to the control group.

Although the animals evaluated have not been subjected to a stress condition, the highest number of hepatocytes verified with the inclusion of the homeopathic complex is important. These cells are the first target in adverse situations like the exposure of the fish to an environment with toxic substances, demonstrating that the homeopathic complex Homeopatila 100® acts in a beneficial way on the liver parameters, demonstrating that the action of this product results in a hepatoprotective effect.

The percentage of glycogen present in hepatocytes was lower in females fed without Homeopatila 100®. The largest amount of this reserve substance in the treatment containing 40 mL of the homeopathic complex per kg of feed was also verified for Nile tilapia (Braccini *et al.*, 2013). Nevertheless, the percentage of glycogen present inside zebrafish hepatocytes was higher than that observed in Nile tilapia.

When fish are well fed, hepatocytes store significant amounts of glycogen and process large amounts of lipids. In this research, fish were given a high-quality commercial diet, being thus, well nourished. Besides, Braccini *et al.* (2013) state that the studied homeopathic complex improves protein absorption and, therefore, provides a higher reserve of glycogen in the fish organism.

The number of eggs was higher for females fed diets containing 40 and 60 mL of Homeopatila 100®, demonstrating the positive effect of the product on this reproductive parameter. The number of eggs is an important factor considered in the production of fish, since the quantity is related to the individuals generated and made available in the productive system. Also, the hatching probability is paramount. We observed higher hatching probability of zebrafish embryos from treatments with 20 and 60 mL of Homeopatila 100® per kg of feed.

The probability of greater hatching in the treatments containing the homeopathic complex may be related to the positive action on the reproductive metabolism of the females. The process of vitellogenesis, in which reserves are stored in oocytes originating the vitello, is essential for embryonic development after fertilization (Levi *et al.*, 2009). Nutrition of breeders influences this stage and the liver plays a key role in reproductive metabolism. Precursors of vitellogenin are synthesized in the liver, secreted into the plasma and transported to the oocytes and later used in vitellogenesis (Levi *et al.*, 2009). According to these authors, during this process, during sexual maturation, there are some metabolic alterations, such as depletion of plasma proteins, calcium, magnesium and glycogen.

In the present study, the females fed Homeopatila 100® had a higher percentage of glycogen in the hepatocytes. Among the evaluated levels, the values were lower for those consuming diet with 20 and 60 mL of the product per kg of diet. On the other hand, the reproduction of individuals of these treatments resulted in a higher probability of hatching of the larvae, and it can be considered that the observed glycogen depletion is related to its use in the vitellogenesis process, reflecting the better development of the embryos.

Homeopathy acts by supporting the body to restore balance after adverse situations, such as stress. Several situations related to the productive system cause stress and affect the balance of fish kept in captivity. They may be of exogenous origins such as management, stocking density and water quality, or endogenous, such as the metabolism involved in gonadal maturation and reproduction.

Stress causes physiological and biochemical imbalance, affecting the productive and reproductive parameters of the fish, including negative action on the liver and the decrease in the quality of gametes (Papadaki *et al*., 2008). Animals reared in conditions of low stress better develop their production potential (Siena *et al*., 2010). In this way, it is prudent to apply measures aimed at the return to homeostasis, which may be using homeopathic products, given the advantages of this therapeutic form.

## CONCLUSIONS

The homeopathic complex Homeopatila 100® acts in a beneficial way in the zebrafish organism, increasing the number of hepatocytes and percentage of glycogen and favoring the reproduction, resulting in higher number of eggs per female and hatching probability of the embryos. It is recommended to use 60 mL of Homeopatila 100® per kg of feed in zebrafish feed.

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