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ANIMAL PRODUCTION

Garlic essential oil increases rates of eggs fertilization and hatching of *Rhamdia quelen* larvae in an artificial incubation system

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ABSTRACT: *The objective of this study was to evaluate the effects of garlic (Allium sativum) essential oil on the rates of eggs fertilization and hatching and on the normal development of larvae of artificially incubated silver catfish (Rhamdia quelen). The experiment was carried out using a completely randomized experimental design with four treatments and five replicates. The treatments consisted of introducing garlic essential oil into the incubators, which used a closed system of water recirculation, at concentrations of 0, 1, 3, and 5mg L^{-1}. Rates of eggs fertilization and larval hatching were determined at 10 and 24h after the beginning of eggs hydration. Morphology of larvae was assessed with the aid of a stereo microscope to determine the rate of normal development. The concentration of 5mg L^{-1} promoted better rates of eggs fertilization and larval hatching. Concentrations of garlic essential oil had a linear effect on eggs fertilization rates and larval hatching. Garlic oil did not influence the morphology of the larvae ($P>0.05$). It is recommended to use 5mg L^{-1} of garlic essential oil to promote better rates of eggs fertilization and larval hatching of artificially incubated silver catfish.*
Key words: *silver catfish, antimicrobials, hatching rate, larvae, Allium sativum.*

Óleo essencial de alho aumenta as taxas de fertilização de ovos e de eclosão de larvas de *Rhamdia quelen* em sistema de incubação artificial

RESUMO: *O objetivo deste trabalho foi avaliar o efeito da utilização do óleo essencial de alho (Allium sativum) sobre as taxas de fertilização dos ovos e eclosão e normalidade de larvas de jundiá cinza, Rhamdia quelen, incubados artificialmente. O experimento foi realizado utilizando delineamento experimental inteiramente casualizado com quatro tratamentos e cinco repetições. Os tratamentos consistiram na introdução de óleo essencial de alho nas incubadoras, utilizando um sistema fechado de recirculação de água, com concentrações de 0; 1; 3 e 5mg L^{-1} de óleo essencial de alho. As taxas de fertilização dos ovos e eclosão das larvas foram determinadas, respectivamente as 10 e 24h após o início da hidratação dos ovos. Utilizou-se do padrão morfológico de larvas para determinar a taxa de normalidade, com o auxílio de um microscópio estereoscópio. A concentração de 5mg L^{-1} promoveu melhores taxas de fertilização de ovos e eclosão de larvas. As concentrações de óleo essencial de alho apresentaram efeito linear sobre as taxas de fertilização dos ovos e eclosão das larvas. O óleo de alho não influenciou na normalidade das larvas ($P>0,05$). Recomenda-se utilizar 5mg L^{-1} de óleo essencial de alho para promover melhores taxas de fertilização de ovos e eclosão de larvas de jundiá cinza incubados artificialmente.*
Palavras-chave: *jundiá cinza, antimicrobianos, taxa de eclosão, larvas, Allium sativum.*

The artificial incubation of fish eggs is indispensable in commercial fish farms in order to increase fingerling production. This technique of reproduction protects the eggs and is independent of uncontrolled environmental conditions, thus providing better egg quality and increasing the number of larvae hatched. The success of this technology depends on good management practices such as the monitoring of incubation water parameters and the elimination of pathogens to avoid large production losses (ANDRADE et al., 2015).

Products such as malachite green, formalin, hydrogen peroxide, potassium permanganate, and iodine are commonly used to combat harmful microorganisms in fish egg incubators (FUANGSAWAT et al., 2011). In fish farms, these chemicals present a risk of contaminating aquatic environments and of having cumulative and carcinogenic effects, as well as of being unreliable, leading to the search for potential substitutes (REVERTER et al., 2014).

Natural products can be an alternative to the above chemicals. Some plants have active

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compounds with antimicrobial, immunostimulating, and nutritional properties and are being used in aquaculture (REVERTER et al., 2014; SYAHIDAH et al., 2015; PEREIRA et al., 2016). Plant-derived products are a promising source of bioactive molecules, while being readily available, less costly, and biocompatible (BULFON et al., 2015). Among these medicinal plants, garlic stands out for having, in its bulb, sulfide compounds such as allicin and ajoene, in addition to bioflavonoids (LEE & GAO, 2012). These compounds have antiviral, antifungal, and antibacterial properties (SANTHOSHA et al., 2013).

The objective of the current study was to evaluate the effects of garlic (*Allium sativum*) essential oil on the rates of eggs fertilization and hatching, and on the normal development of larvae of artificially incubated silver catfish (*Rhamdia quelen*).

An egg incubation structure with four individual systems of water recirculation was developed to carry out this study. In each system, the water circulated from the top (4 × 250L reservoirs) to five 2.5L PET bottles that were adapted as Zuger-type incubators. The study was carried out in December 2014. The completely randomized experimental design consisted of four treatments (0, 1, 3, and 5mg L^{-1} of garlic essential oil), each with five replicates (incubators). Garlic essential oil (LASZLO Aromatologia Ltda., Belo Horizonte, MG, Brazil), consisting of about 60% diallyl disulfide and 20% diallyl trisulfide, was added to the three treatments one hour before transferring the eggs to the incubators used in each of the water recirculation systems.

During the incubation period, pH (7.4 to 7.8), dissolved oxygen (6.8 to 7.7mg L^{-1}), and water temperature (23.5 to 25.1°C) were measured in the morning and in the afternoon using a digital oximeter, a dissolved oxygen meter (YSI 550A-DO; YSI Environmental, Yellow Springs, USA), and a pH meter (HI 8314; Hanna Instruments®). The values of these water quality parameters remained within the recommended standards for artificial incubation of silver catfish eggs (BOMBARDELLI et al., 2006).

For reproductive manipulation, males that released semen when subjected to mild abdominal pressure and females that demonstrated a rounded abdomen, reddish urogenital papillae, and oocytes with uniform color and size were randomly selected from the breeding tanks (ANDRADE et al., 2015). The selected breeders were individually weighed, marked, and separated by sex in two tanks, each equipped with constant aeration and water renewal. Twelve silver catfish were used to collect gametes; the seven females had a mean weight of 424±182g and the five males 211±98g.

Hormonal manipulation was carried out using carp pituitary extract (CPE). The protocol used for females consisted of 5.5mg CPE kg^{-1} distributed in two doses, 10% in the first application and the rest 12 hours later (BOMBARDELLI et al., 2006). The oocytes were collected from each of the females by means of abdominal massage, 240 degree-hours (10h, water at 24°C) from the second hormonal application. For the male silver catfish, a single dose of 3.0mg CPE kg^{-1} was applied, and the semen was removed by means of abdominal massage in the cephalocaudal direction after 240 degree-hours. The semen was collected in test tubes and kept cool (12°C) for analysis of sperm and seminal parameters (SANCHES et al., 2011).

Oocytes were fertilized with 50µL semen and activated with 20mL water. Fertilized eggs were rinsed three times with water from the system itself and 2.0mL oocytes were transferred to each of the 20 incubators. For estimating the number of oocytes in each incubator, three samples of approximately 0.1mL of oocytes each were used for counting and for estimation of the number of oocytes in 2.0mL, according to procedures adapted from BOMBARDELLI et al. (2006).

Fertilization rates were calculated after blastopore closure, approximately 10 hours after fertilization. For this, a sample was collected from each incubator using a pipette and transferred to a graduated test tube with approximately 1mL of eggs. The relationship between the number of fertilized (viable) eggs and the total number of eggs of each sample expressed as percentage (fertilization rate = number of viable eggs × 100/total number of eggs) was determined. Embryonated eggs in the blastopore closure stage were considered viable (GODINHO, 2007).

After larvae hatched-after approximately 24h of incubation-larval hatching and rates of normal development were determined. All larvae and non-hatched eggs were collected from each experimental unit. They were retrieved with the aid of a sieve and fixed in 4% buffered formalin for subsequent larval counting and morphological analysis. Hatching rates (%) were determined as the number of hatched larvae with respect to the total number of eggs (GODINHO, 2007). Subsequently, the percentage of normal larvae was estimated from the total of fixed larvae using a stereo microscope (4× objective) to determine normal development.

Results of the rates (%) of eggs fertilization, hatching, and normal development of silver catfish larvae were submitted to normality tests and analysis of variance (ANOVA). The means were compared by the Dunnett test (5%). Regression analysis was applied at 5% of probability, using the statistical software SAEG (System of Statistical and Genetic Analysis).

Concentrations of garlic essential oil demonstrated an increasing linear effect on egg fertilization and hatching rates of silver catfish larvae ($P<0.05$). The addition of garlic oil in the water of the incubators did not influence ($P>0.05$) larval morphology (Table 1). Concentration of 5mg L^{-1} garlic essential oil resulted in improved eggs fertilization and larval hatching rates, with averages of 74.86 and 66.21% respectively. Both rates were significantly different ($P<0.05$) than those of the control (Table 1). Results obtained here corroborated the research of MOUSAVI et al. (2009), which reported a 70% hatching rate with a 10mg L^{-1} concentration of a blend of essential oils obtained from different medicinal plants (*Thymus vulgaris*, *Salvia officinalis*, *Eucalyptus globulus*, and *Mentha piperita*) in rainbow trout (*Oncorhynchus mykiss*) egg incubators.

The positive effects of garlic essential oil at 5mg L^{-1} may have resulted from the improvement of water microbiological parameters; garlic is currently used in aquaculture for its several active components that act as antimicrobials. Sulfur compounds, such as allicin and ajoene, act against parasites in the prevention and cure of diseases (LEE & GAO, 2012) and as immunostimulants and antimicrobials (SANTHOSHA et al., 2013).

Despite the wide margin of safety in the use of medicinal plant extracts, there are also a few reports on their negative impacts on fish farming (SYAHIDAH et al., 2015). Some products may cause damage to the membranes of embryonated eggs. Essential oils concentrate many products of plant secondary metabolism and may be toxic. In the essential oils of garlic, the most toxic components are diallyl disulfide and diallyl trisulfide (ZHAO et al., 2013), which are products of the degradation of allicin. These sulfur compounds are also bioactive compounds of interest, as they are co-responsible for the antimicrobial activity of garlic oil (BULFON et al., 2015; PEREIRA et al., 2016). However, if used at very high concentrations, these compounds lead to mortality of both eggs and larvae. In a study on the lethality of garlic extract (a product less concentrated than essential oil) on carp eggs and larvae, ABDEL-HADI et al. (2008) reported that a concentration of 0.5g L^{-1} was not harmful for eggs, but was lethal for larvae.

Besides improving the productive aspects in the reproduction of silver catfish under artificial incubation, garlic essential oil may have relevance in terms of environmental sustainability. Garlic oil can replace synthetic compounds, with the advantage of having a lower potential for environmental damage. Further studies should be conducted to analyze the effects of garlic essential oil on bacterial load and fungi during egg incubation and larval farming.

From the results obtained in the current study, it is concluded that the addition of garlic essential oil in the water at a concentration of 5mg L^{-1} promotes an increase in the eggs fertilization and hatching rates of artificially incubated silver catfish larvae in a water recirculation system.

Table 1 - The rates (mean ± standard deviation) of eggs fertilization and hatching and on the normal development of larvae of artificially incubated silver catfish under different concentrations of garlic essential oil.

Garlic essential oil (mg L^{-1})	Eggs fertilization (%)	Larval hatching (%)	Larval normality (%)
0 (control)	57.22 ± 6.46	47.28 ± 5.19	49.70 ± 17.31
1	55.83 ± 9.15^{ns}	48.06 ± 9.56^{ns}	44.55 ± 4.69^{ns}
3	62.98 ± 13.75^{ns}	50.26 ± 5.25^{ns}	54.18± 7.72^{ns}
5	74.86 ± 6.53*	66.21 ± 5.44**	43.14 ± 6.85^{ns}

Eggs fertilization = 54.3940 + 3.7006x, R^2 = 0.8965; larval hatching = 44.8190 + 3.6147x, R^2 = 0.8060. Ns (non-significant). The asterisks represent statistical difference between means, in relation to the control group, by the Dunnett test with $P<0.05$ (*) or $P<0.01$ (**).

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

Protocol Nº. 03112/2012, Ethics Committee on Animals Use (CEUA)/Universidade Estadual do Oeste do Paraná (Unioeste).

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REFERENCES

ABDEL-HADI, Y.M. et al. Study on the use of *Artemisia cina* L. (wormseed plants) and *Allium sativum* (garlic) in the control of Saprolegniosis in egg of *Cyprinus carpio* (common carp) and *Hypophthalmichthys molitrix* (silver carp). In: MALAYSIAN SYMPOSIUM ON MICROBIOLOGY (MSM), 30., 2008, Kuantan. **Proceedings…** Kuantan, Malaysia: MSM, 2008. p.571-573.

ANDRADE, E.S. et al. Reproductive biology of freshwater fish. **Revista Brasileira de Reprodução Animal**, v.39, n.1, p.195-201, jan./mar. 2015. Available from: <http://revistas.bvs-vet.org.br/rbra/article/view/26708>. Accessed: Mar. 19, 2017.

BOMBARDELLI, R.A. et al. Insemination dose for artificial fertilization of grey jundia oocytes, *Rhamdia quelen* (Quoy & Gaimardm, 1824). **Revista Brasileira de Zootecnia**, v.35, n.4, p.1251-1257, aug. 2006. Available from: <http://www.scielo.br/pdf/rbz/v35n4/01.pdf>. Accessed: Mar. 18, 2017. doi: 10.1590/S1516-35982006000500001.

BULFON, C. et al. Current research on the use of plant-derived products in farmed fish. **Aquaculture Research**, v.46, n.3, p.513-551, mar. 2015. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/are.12238/full>. Accessed: Mar. 19, 2017. doi: 10.1111/are.12238.

FUANGSAWAT, W. et al. Sensitivity comparison of pathogenic aquatic fungal hyphae to sodium chloride, hydrogen peroxide, acetic acid and povidone iodine. **Kasetsart Journal of Natural Science**, v.45, n.1, p.84-89, jan./fev. 2011. Available from: <http://kasetsartjournal.ku.ac.th/kuj_files/2011/A1103021414078125.pdf>. Accessed: Mar. 17, 2017.

GODINHO, H.P. Reproductive strategies of fishes applied to aquaculture: bases for development of production technologies. **Revista Brasileira de Reprodução Animal**, v.31, n.3, p.351-360, jul./set. 2007. Available from: <http://cbra.org.br/pages/publicacoes/rbra/download/351.pdf>. Accessed: Mar. 19, 2017.

LEE, J.Y.; GAO, Y. Review of the application of garlic, *Allium sativum*, in aquaculture. **Journal of the World Aquaculture Society**, v.43, n.4, p.447-458, aug. 2012. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/j.1749-7345.2012.00581.x/full>. Accessed: Mar. 18, 2017. doi: 10.1111/j.1749-7345.2012.00581.x.

MOUSAVI, S.M. et al. Evaluation of activity of new combined essential oils in comparison with malachite green on hatching rate in rainbow trout (*Oncorhynchus mykiss*) eggs. **Journal of Fisheries and Aquatic Science**, v.4, n.2, p.103-110, mar./abr. 2009. Available from: <http://scialert.net/fulltex/?doi=jfas.2009.103.110>. Accessed: Mar. 18, 2017. doi: 10.3923/jfas.2009.103.110.

PEREIRA, L.A. et al. Use of plant extracts and their prophylactic or therapeutic properties in the fish production. **Scientia Agraria Paranaensis**, v.15, n.4, p.1-10, out./dez. 2016. Available from: <http://dx.doi.org/10.18188/1983-1471/sap.v15n4p373-380>. Accessed: Mar. 19, 2017. doi: 10.18188/1983-1471/sap.v15n4p373-380.

REVERTER, M. et al. Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives. **Aquaculture**, v.433, p.50-61, sep. 2014. Available from: <http://www.sciencedirect.com/science/article/pii/S0044848614002798>. Accessed: Mar. 18, 2017. doi: 10.1016/j.aquaculture.2014.05.048.

SANCHES, E.A. et al. Artificial fertilization of oocytes and sperm activation in pacu: effects of the spermatozoa: oocyte ratio, water volume, and *in natura* semen preservation. **Revista Brasileira de Zootecnia**, v.40, n.1, p.1-6, jan. 2011. Available from: <http://www.scielo.br/pdf/rbz/v40n1/v40n1a01.pdf>. Accessed: Mar. 19, 2017. doi: 10.1590/S1516-35982011000100001.

SANTHOSHA, S.G. et al. Bioactive components of garlic and their physiological role in health maintenance: a review. **Food Bioscience**, v.3, n.9, p.59-74, sep. 2013. Available from: <http://www.sciencedirect.com/science/article/pii/S2212429213000473>. Accessed: Mar. 18, 2017. doi: 10.1016/j.fbio.2013.07.001.

SYAHIDAH, A. et al. Status and potential of herbal applications in aquaculture: a review. **Iranian Journal of Fisheries Sciences**, v.14, n.1, 27-44, jan./jul. 2015. Available from: <http://jifro.ir/article-1-1818-en.html>. Accessed: Mar. 19, 2017.

ZHAO, N.N. et al. Evaluation of acute toxicity of essential oil of garlic (*Allium sativum*) and its selected major constituent compounds against overwintering *Cacopsylla chinensis* Hemiptera: Psyllidae). **Journal of Economic Entomology**, v.106, n.3, p.1349-1354, jun. 2013. Available from: <http://dx.doi.org/10.1603/EC12191>. Accessed: Mar. 18, 2017. doi: 10.1603/EC12191.