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Application of rosemary essential oil (*Rosmarinus officinalis* Linnaeus, 1753) for anesthesia and transport of bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) fingerlings

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Abstract. *The aim of the present research is to study the efficacy of rosemary oil as an anesthetic for bighead carp (Hypophthalmichthys nobilis Richardson, 1845) and as an anesthetic used for transport of fingerlings. The fish used in the experiment have an average body weight (BW, g) of 42.13±17.90 and an average total length (TL, cm) of 16.54±2.32. Five treatments are conducted with five experimental concentrations: 0.20 ml.l^{-1}, 0.30 ml.l^{-1}, 0.40 ml.l^{-1}, 0.50 ml.l^{-1} and 0.60 ml.l^{-1}. For each concentration 10 fish are used or a total of 50 fish. For the transport experiment, 3 concentrations are used: 0.06 ml.l^{-1}, 0.08 ml.l^{-1} and 0.10 ml.l^{-1}. The applied stocking density is 3 fish/l. A total of 60 fish are used for each concentration or a total of 180 fish. The established results, for the first experiment, show that at concentration of 0.60 ml.l^{-1} the induction of anesthesia is the fastest (3.46 min). From all tested concentrations the recovery time is the shortest at the lowest concentration – 2.16 min ($p \leq 0.001$). The concentration of 0.10 ml.l^{-1} of rosemary oil can be used for transport of bighead carp fingerlings for a period from 1 to 2 hours. The concentration of 0.08 ml.l^{-1} can be applied when the duration of the transport is from 1 to 3 hours.*

Keywords: anesthesia, bighead carp, *Hypophthalmichthys nobilis*, *Rosmarinus officinalis*, rosemary oil, transport

Introduction

Anesthesia is used in fish farming as a method to reduce stress and prevent fish from injury when performing various manipulations, such as artificial reproduction, vaccination, surgery, etc. (Hamachkova et al., 2006; Velisek et al., 2006; Zaikov et al., 2008; Brown, 2011; Mikodina et al., 2011). According to Coyle et al. (2004) and Brown (2011), a good anesthetic in aquaculture must meet the following basic requirements: to induce rapid anesthesia within 1-5 minutes with minimal stress of the treated species, no persistent side effects on the physiology and behavior of the fish, rapid recovery from anesthesia, efficacy at low doses, low cost and affordability. Woody et al. (2002) and Ross and Ross (2008) state that the properties addressed for choosing an efficient anesthetic agent for fish will vary according to the planned activities and the species, but in general fast induction (within 3 min), fast recovery (10 min after 15 min exposure time) and no subsequent mortality are among the most important criteria considered to guarantee safe process of anesthesia.

Common rosemary (*Rosmarinus officinalis* L.) is a perennial herbaceous plant with evergreen coniferous leaves, belonging to family *Lamiaceae*. The plant is native to the Mediterranean region, but it is currently found in all parts of the world (European Medicines Agency, 2010; Gonzales-Minero, 2020). The main active ingredients of rosemary essential oil are alpha-pinene and eucalyptol (1.8-cineole) (Bauer et al., 1997).

Rosemary essential oil has not been researched as an anesthetic for bighead carp (*Hypophthalmichthys nobilis*, Richardson, 1845) or for other fish species. It has been used only for anesthesia of carp, *Cyprinus carpio*, Linneius 1758, (mean weight = 652 g) with concentrations from 0.25 to 1 ml.l^{-1} (Ghazilou and Chenary, 2011). *R. officinalis* has been applied as feed additive for different fish species, such as rainbow trout, *Oncorhynchus mykiss* (Glenn et al., 2014), gilthead seabream, *Sparus aurata* (Hernandez et al., 2015), African catfish, *Clarias griepinus* (Turan and Yigitarslan, 2016) and Nile tilapia, *Oreochromis niloticus* (Yilmaz et al., 2019), showing positive effect on fish growth and feed utilization.

There are various studies of the anesthetic effect of different essential oils, such as lavender, *Lavandula angustifolia* (Metin et al., 2015; Can and Sumer, 2019), *Lavandula hybrida* (Can et al., 2019), *Lavandula angustifolia* (Alshkarchy et al., 2020), basil, *Ocimum basilicum* (Khumpirapang et al., 2018), poppy plant, *Papaver nudicaule* (Al-hamadani et al., 2020) and spearmint, *Mentha spicata* (Roohi and Imanpoor, 2015).

One way to avoid handling related stress is to subject fish to temporary anesthesia. A study of Juell (2013) evaluated the efficacy of three anesthetics - MS-222, Benzoak and Aqui-Sfor silver carp, *Hypophthalmichthys molitrix* (Valenciennes, 1844), fingerlings. The author also examined the stress response by measuring plasma cortisol and glucose in after

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temporary water level reduction. Induction time, recovery time and tolerance to prolonged exposure are tested for each of the anesthetics. Only one experiment has been conducted by Akbary et al. (2016) investigating the stress caused in bighead carp, *Hypophthalmichthys nobilis* (Richardson, 1845) (BW=65.4 g) during anesthesia. The authors examined the level of cortisol and hematological parameters after anesthesia with 2-phenoxyethanol.

Up to date no studies have been performed regarding the transport of bighead carp. Research on the use of anesthetics (essential oils or chemical agents) in the transport of live fish is very scarce (Benovit et al., 2012; Tondolo et al., 2013; Balamurugan et al., 2016; Sampaio and Freire, 2016; Zhao et al., 2017) despite the fact that this is main and important activity in aquaculture. Transported fish can die from shock caused by stress during transport (Bulgarian Food Safety Agency, 2011). In this regard, the addition of an anesthetic with a certain concentration in the transport tank could relieve stress and prevent mortality.

Zhao et al. (2017) conducted an experiment with eugenol by examining its effect on grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844), by treating fish (1.5 kg) at different doses and exposures. The experiment also included taking blood samples to examine the residual concentration of eugenol in the blood plasma, muscles and liver. The aim of the experiment was to determine the most appropriate concentration of eugenol for transport of grass carp.

The aim of the present study is to investigate the anesthetic effect of rosemary essential oil, as well as its application in the transport of fingerlings of bighead carp, as up to date there is no data on the anesthesia of bighead carp with *R. officinalis* oil, nor have any experiments been performed regarding transport of fish by adding this essential oil.

Material and methods

The study was conducted at the Institute of Fisheries and Aquaculture, Plovdiv in March 2021.

Research subject

The subject of the experiment was *H. nobilis* fingerlings hatched by artificial propagation in May 2020 and afterwards grown in the ponds in the experimental base of IFA, Plovdiv. For the purpose of the study, the fish were caught from the experimental ponds and were imported for storage in 3 m^3 tanks.

Essential oil

The *R. officinalis* essential oil was purchased commercially, with listed ingredients 100% pure rosemary oil, produced in Plovdiv, Bulgaria by "Rivana" LTD. The experimental solutions were prepared by diluting the oil in ethyl alcohol (95%) in 1:9 ratio and were added to 10 L experimental tanks with vigorous stirring before treatment.

Preliminary test

Due to the lack of sufficient data, a preliminary test was performed to study different concentrations of rosemary oil. Two separate groups of 5 fish were treated twice, since the size of the fish allowed observations of more than one specimen.

In order to preserve the well-being of the treated fish and to prevent mortality, the lowest experimental concentration was 0.02 $ml.l^{-1}$. The preliminary test was performed with 7 experimental concentrations: 0.02 $ml.l^{-1}$, 0.04 $ml.l^{-1}$, 0.06 $ml.l^{-1}$, 0.08 $ml.l^{-1}$, 0.10 $ml.l^{-1}$, 0.12 $ml.l^{-1}$, and 0.14 $ml.l^{-1}$, with an exposure of 20 min so that the effect of the oil can be monitored for a longer period of time.

Experiment 1 – anesthetic effect of R. officinalis

Based on the preliminary test, 5 experimental concentrations were used in the study of the anesthetic effect of rosemary oil: 0.20 $ml.l^{-1}$, 0.30 $ml.l^{-1}$, 0.40 $ml.l^{-1}$, 0.50 $ml.l^{-1}$ and 0.60 $ml.l^{-1}$. Two separate groups of 5 fish were treated twice in order to obtain statistical reliability of the results. For each concentration, 10 fish were used or a total of 50 fish for the experiment. The biometric parameters, body weight (BW, g) and body length (TL, cm), are presented in Table 1.

Table 1. Body weight (BW, g) and total length (TL, cm) of the fish

Statistical value	BW, g	TL, cm
mean±SD	42.13±17.90	16.54±2.32
lim	15.79÷79.06	12.70÷21.80
CV, %	42.49	14.02

When preparing the solutions for anesthesia and recovery, the temperature of the water was equalized to the temperature of the water in the storage tanks. Before adding the anesthetic solution, the temperature (T°C) and the level of dissolved oxygen (O_2, $mg.l^{-1}$) were measured. To recover from anesthesia, the fish were transferred in tanks with the same volume of clean water (10 L) with placed microcompressors, where they were observed until complete recovery. The time required for the induction of anesthesia and subsequent recovery was measured with a stopwatch, taking into account the time of each phase. When processing the results, the data were converted into minutes according to the following formula:

Minutes = (minutes*60 + seconds / 60).

The behavior of the fish was described and analyzed according to the phases of anesthesia and recovery determined by Hamackova et al. (2006):

Phases of anesthesia:

Phase 1. Accelleration of the opercular movements, increased respiratory activity;

Phase 2. Decreased respiratory activity accompanied by uncoordinated movements;

Phase 3. Loss of equilibrium, decreased opercular movements, the fish still react to strong external stimuli;

Phase 4. Complete immobilization, the fish lie on the bottom and do not react to handling.

Phases of recovery:

Phase 1. Beginning of movements;

Phase 2. Weak, uncoordinated locomotor activity;

Phase 3. Normal position of the body. Normal locomotor activity is regained.

Experiment 2 - transport trial

The experiment for transport was carried out in laboratory conditions according to the "Instruction for the application of the requirements for the transport of live fish" (BFSA, 2011). Prior to the beginning of the experiment, the fish were not fed for 24 hours and an assessment of their health was made. The experiment was conducted with three variants of concentrations of rosemary oil, in 10 L tanks, based on the preliminary test: Variant 1 - 0.06 ml.l^{-1}, Variant 2 - 0.08 ml.l^{-1} and Variant 3 - 0.10 ml.l^{-1}. For each variant 30 fish were used or a total of 90 fish. Microcompressors were placed in the water 1 hour before the introduction of the fish in the experimental variants. Hydrochemical measurements (T°C and O_2 mg.l^{-1}) were performed every hour during the experiment. The applied density was 3 fish/l, calculated on the basis of the average weight of the fish according to the norms indicated in the instruction presented in Table 2.

Table 2. Requirements for density and duration of transport of herbivorous fish

Fish species	Density	Duration of transport, hours
Herbivorous fish		
Density	70 - 130 kg/m³ depending on the weight	from 3 to 6
The amount of fish with an average body weight below 100 g can be increased by 60-80%		

Source: Instruction for application of the requirements for transport of live fish (BFSA, 2011).

The behavior of the fish in the three variants was observed every hour according to the table of Hamackova et al. (2006). The fish in each phase were counted and their percentage was calculated according to their total number for each variant. The following formula was used:

Phase, % = (Number of fish in the phase*100) / Total number of fish.

At the end of the experiment, the water from the tanks was drained slowly so that the fish did not stay dry. Immediately after that, clean water of the same volume and temperature was added. After 3 to 5 minutes the fish were released into their natural environment.

Statistical analysis

The results obtained for the induction of anesthesia and the period of recovery, for each concentration and phase, were analyzed at a confidence level of p≤0.05. For this purpose, a comparative Student T-test was performed using Excel - Data analysis.

Results and discussion

At all concentrations studied in the preliminary test (0.02 ml.l^{-1}, 0.04 ml.l^{-1}, 0.06 ml.l^{-1}, 0.08 ml.l^{-1}, 0.10 ml.l^{-1}, 0.12 ml.l^{-1}, and 0.14 ml.l^{-1}) the fish reached only Phase 2 of the anesthesia (Figure 1).

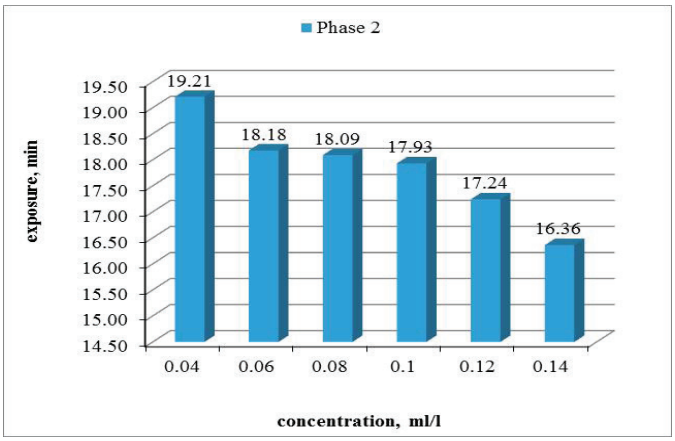

Figure 1. Time to reach Phase 2 at different experimental concentrations during preliminary test

In Phase 2 the fish remain static, do not move or perform very slow, uncoordinated movements. The fish enter Phase 2 in 16.36 min at the highest concentration of 0.14 ml.l^{-1} and in 19.21 min at 0.04 ml.l^{-1}. At the lowest concentration of 0.02 ml.l^{-1}, the fish swim normally during the entire exposure of 20 minutes, therefore this concentration is not included in the graph. A recovery process is not observed at any of the experimental concentrations in the preliminary test as the fish did not reach anesthesia phase.

The results of the anesthesia with the experimental concentrations (0.20 ml.l^{-1}, 0.30 ml.l^{-1}, 0.40 ml.l^{-1}, 0.50 ml.l^{-1} and 0.60 ml.l^{-1}) are presented in Table 3.

Table 3. Duration (min) of the phases of anesthesia and recovery of bighead carp fingerlings (T1)

Anesthesia and recovery phases	Concentration (ml.l^{-1})					p
	0.20	0.30	0.40	0.50	0.60	
A2- Phase 2 of anesthesia	10.49±1.96^{ac}	7.49±2.29^{bd}	3.01±0.42^{cb}	2.07±0.69^{da}	0.99±0.24^{ec}	***
A3- Phase 3 of anesthesia	17.64±0.76^{a}	12.15±2.50^{a}	6.92±0.80^{a}	-	-	ns
A4- Phase 4 of anesthesia	19.25±0.38^{a}	13.59±2.79^{a}	7.15±0.73^{a}	4.51±1.05^{ba}	3.46±0.52^{ca}	*
R1- Phase 1 of recovery	1.53±0.74^{ae}	2.61±0.56^{b}	2.95±0.48^{cd}	3.10±0.48^{dc}	3.86±0.48^{ea}	***
R2- Phase 2 of recovery	1.75±0.60^{ad}	2.79±0.51^{bd}	3.11±0.47^{ce}	3.33±0.45^{dc}	4.87±0.63^{dd}	**
R3- Phase 3 of recovery	2.16±0.60^{ad}	3.39±0.43^{b}	3.66±0.40^{cd}	3.88±0.34^{d}	5.66±0.78^{dd}	***

Values connected by different superscripts are significantly different (p≤0.05); ***p≤0.001; **p≤0.01; *p≤0.05; ns- no significant difference.

At 0.20 ml.l^{-1}, 50% of the fish are anesthetized, the rest reach Phase 3. A recovery process is observed with the fish that do not reach Phase 4 of anesthesia, although it is shorter. At a concentration of 0.30 ml.l^{-1}, as well as at subsequent concentrations, the fish react to the solution with rapid movements, which gradually subside. At the highest

concentrations of 0.50 ml.l^{-1} and 0.60 ml.l^{-1}, the fish enter directly from Phase 2 of the uncoordinated movements to Phase 4 of anesthesia, skipping Phase 3. This is expressed in the following behavior: the fish perform very slow, uncoordinated movements, which gradually slow down and the fish lie on the bottom under full anesthesia. Phase 4 occurs faster at 0.60 ml.l^{-1} than at 0.50 ml.l^{-1} ($p \leq 0.05$).

Phase 1 of recovery occurred fastest at 0.20ml.l^{-1} and 0.30ml.l^{-1} ($p \leq 0.001$), while at other concentrations the fish began movements later ($p \leq 0.001$). Thus, Phase 2 occured fastest at 0.20 ml.l^{-1} and slowest at 0.60 ml.l^{-1} ($p \leq 0.01$). Phase 3 of complete recovery occurred in the shortest time interval at 0.20ml.l^{-1} compared to all other concentrations ($p \leq 0.001$).

The results of the experiment of Ghazilou and Chenary (2011) with *C. carpio*, with an average weight of 625 g, show that at experimental rosemary essential oil concentrations from 0.25 to 1.00 ml.l^{-1} Phase 4 of complete anesthesia is achieved within 3 min.The authors report that an increase in concentration resulted in a significant shortening of anesthesia induction time, which is also confirmed in the current study.

Comparing the obtained results from both studies, it can be concluded that in the present study Phase 4 of anesthesia is achieved in 3.46 min at the highest concentration of 0.6 ml.l^{-1}, while in the study of Ghazilou and Chenary (2011) anesthesia occured in 28 sec at 1 ml.l^{-1}.At 0.50 ml.l^{-1}, Ghazilou and Chenary (2011) achieved anesthesia in 40 sec, while in the present study, at the same concentration of rosemary oil, anesthesia of bighead carp fingerlings occurred six times slower - for 4.51 min. The reason for the different effect of rosemary oil may be due to a range of factors, such as fish species, different stage of individual development, different methods of preparation of the anesthetic solution, etc.

Juell (2013) conducted a study with three synthetic agents: MS-222, Benzoak and Aqui-S with fingerlings of bighead carp (BW=54.7 g). The experimental concentrations of MS-222 and Benzoak were 0.025, 0.050, 0.075 and 0.10 ml.l^{-1}, while with Aqui-S - 0.01, 0.02, 0.05 and 0.10 ml.l^{-1}. From the comparison of the results it is obvious that at a concentration of 0.10 ml.l^{-1} anesthesia is achieved in the range from 3.9 min to 1.3 min, while at twice higher concentration of rosemary oil (0.20 ml.l^{-1}) anesthesia occured in 19.25 minutes in 50% of fish. Therefore, those synthetic anesthetics MS-222, Benzoak and Aqui-S at low concentrations are more effective than rosemary oil.

The main hydrochemical parameters are presented in Table 4.

Table 4. Main hydrochemical parameters of the water in the tanks for anesthesia and recovery

Statistical value	Anesthesia bath		Recovery bath	
	O_2 mg.l^{-1}	T, ºC	O_2 mg/l^{-1}	T, ºC
mean±SD	4.48±0.48	19.60±0.63	7.30±0.44	19.30±0.80
min	3.80	18.80	6.80	18.00
max	5.00	20.40	7.80	20.10

The temperature in the tank for anesthesia is 1.5% higher than the temperature in the tank for recovery and the minimum and maximum values vary within narrow limits. In contrast to temperature, the oxygen values are lower in the tank for anesthesia than in the tank for recovery. The reason for this are the installed microcompressors which support the recovery process.

During the transport experiment, the behavior of the fish varied between normal motor activity and Phase 2 of the anesthesia, expressed in decreased locomotor activity and slow uncoordinated movements. Phase 3 of loss of balance occured in Variant 3 (0.10 ml.l^{-1}) after 3 hours and it is observed only in 6% of the fish. Upon external stimuli the fish immediately regain their normal body position (Table 5).

Table 5. Results of the experiment for transport of bighead carp fingerlings with rosemary oil in three experimental variants

Hour	Variant 1 (0.06 ml.l^{-1})		Variant 2 (0.08 ml.l^{-1})		Variant 3 (0.10 ml.l^{-1})		
	Phase 0	Phase 2	Phase 0	Phase 2	Phase 0	Phase 2	Phase 3
1	64%	36%	40%	60%	30%	70%	-
2	40%	60%	20%	80%	14%	86%	-
3	35%	65%	10%	90%	-	94%	6%

*The number of fish in the different conditions and phases of anesthesia is represented as a percentage; Phase 0 – normal locomotor activity; Phase 2 – uncoordinated movements (static position); Phase 3 – loss of equilibrium.

The results show that as the transport time increases, the number of fish in a state of normal motor activity decreases in relation to the number of fish that have entered Phase 2 of uncoordinated movements which increases. This trend is observed in all experimental variants.

Based on the conducted experiment, it can be recommended when transporting fingerlings of bighead carp for 1 hour to apply concentration of 0.10 ml.l^{-1}, with the highest percentage of fish with decreased locomotor activity. The concentrations of 0.08ml.l^{-1} and 0.10 ml.l^{-1} can be used for transport of bighead carp from 1 to 2 hours, with concentration of 0.08 ml.l^{-1} being applied for transport from 2 to 3 hours. The highest percentage of fish is in Phase 2, without any fish entering Phase 3 of loss of equilibrium. At a concentration of 0.06 ml.l^{-1}, the percentage of fish in a state of normal motor activity remains the highest in all three variants.

A similar experiment was performed by Zhao et al. (2017) with grass carp and the synthetic substance eugenol at concentrations of 0.005 ml.l^{-1}, 0.01 ml.l^{-1}, 0.015 ml.l^{-1}, 0.02 ml.l^{-1}, 0.03 ml.l^{-1} and 0.04 ml.l^{-1} and exposure for 8 hours, with the aim being to determine the minimum eugenol concentration that keeps the fish in deep sedation without causing mortality and stress. The results obtained show that the optimal concentration is 0.010 ml.l^{-1}, which induced rapid anesthesia with minimum stress within 10 min and maintained deep sedation for 8 hours. The fish then return to normal activity in a few minutes with zero

mortality. At concentration of 0.040 ml.l^{-1} eugenol the authors registered 100% mortality.

Benovit et al. (2012) conducted an experiment for transport of flounder, *Paralichthys orbignyanus* Valenciennes, 1839, using whitebrush, *Aloysia gratissima* and clove basil, *Ocimum gratissimum* as anesthetics. The study found that *A. gratissima* cannot be used for anesthesia or sedation of *P. orbignyanus* due to side effects and mortality. *O. gratissimum* effectively induce anesthesia at 0.05 and 0.1 ml.l^{-1} and can be used for transport of *P. orbignyanus* at concentration of 0.01 ml.l^{-1}.

Tondolo et al. (2013) study the anesthetic effect of *Nectandra megapotamica* in fat snook, *Centropomus paralellus*, but the authors conclude that the application of the essential oil did not prevent the stress from anesthesia and transport. In another research, Balamurugan et al. (2016) have established that the most effective concentration of clove oil for transport of clown fish is 0.0175 ml.l^{-1}.

In the present study, the higher experimental concentrations of rosemary oil (0.08 ml.l^{-1} and 0.10 ml.l^{-1}) are the most effective for transport of bighead carp up to 3 hours, while the established by Zhao et al. (2017) optimal concentration of eugenol is eight times lower (0.01 ml.l^{-1}).

The hydrochemical measurements are presented in Figure 2 and Figure 3.

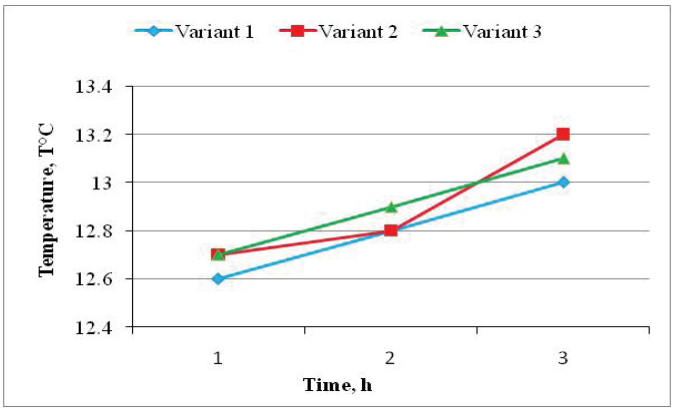

Figure 2. Measured temperature per hour in the three experimental variants

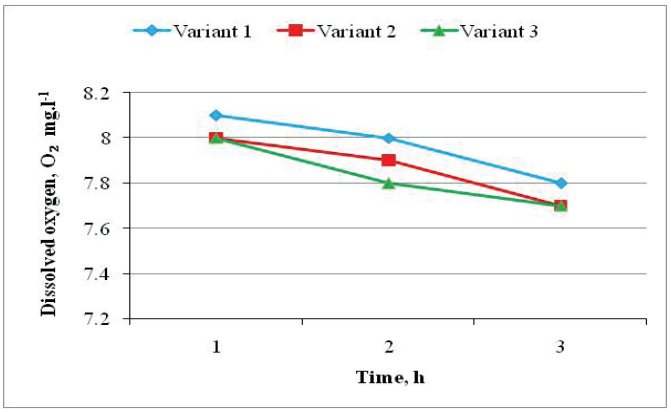

Figure 3. Amount of dissolved oxygen per hour in the three experimental variants

The measured temperature varies from 12.6°C to 13.2°C. According to the official instructions of Bulgarian Food Safety Agency (BFSA), temperature should not exceed 21°C. The amount of dissolved oxygen is in the range of 7.7-8.1 mg.l^{-1} which is within the permissible values according to BFSA.

Conclusion

Based on the results it can be concluded that concentrations from 0.02 ml.l^{-1} to 0.14 ml.l^{-1} of rosemary essential oil, with an exposure of 20 min, have no anesthetic effect on bighead carp fingerlings. As the concentration increases, the time required for anesthesia decreases, with the fastest induction time of anesthesia and slowest period of recovery being observed at the highest concentrations of 0.50 ml.l^{-1} and 0.60 ml.l^{-1}. For transport of bighead carp fingerlings for 1 hour, the optimal concentration of rosemary oil with sedative effect is 0.10 ml.l^{-1}, at which the highest percentage of fish is in a state of decreased locomotor activity. For 1 to 2 hour transport, the concentrations 0.08 ml.l^{-1} and 0.10 ml.l^{-1} can be used. For transport from 2 to 3 hours, the most optimal concentration is 0.08 ml.l^{-1}, at which the highest percentage of fish remain in Phase 2 and no fish enter Phase 3 of loss of equilibrium.

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