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ANESTHETIC EFFICACY OF CLOVE OIL AND 2-PHENOXYETHANOL ON DOCTOR FISH, *Garra rufa* (HECKEL, 1843)

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ABSTRACT

This study aimed to determine the anesthetic efficacy of clove oil and 2-phenoxyethanol on doctor fish (*Garra rufa*) at two different water temperatures. Experimental fish (1.2 ± 0.2 g mean weight) were subjected to 25, 50, 75 and 100 µL L^{-1} clove oil and 100, 200, 300, 400 and 500 µL L^{-1} 2-phenoxyethanol concentrations at water temperature of 15 and 25 °C, and the induction and recovery times were investigated. Results showed that induction and recovery times in doctor fish were significantly affected by clove oil and 2-phenoxyethanol concentrations as well as water temperature. The interaction of anesthetic concentration and water temperature on all induction stage time was significant in clove oil. Between the anesthetic concentration and temperature interaction was significant for recovery times in both anesthetic agents. The induction time decreased significantly with increasing concentration of both anesthetic agents at water temperature of 15 and 25 °C. The lowest effective concentrations that produced induction within 3 min and recovery within 5 min were 50-75 µL L^{-1} of clove oil and 300 µL L^{-1} of 2-phenoxyethanol in both 15 and 25 °C respectively. The results also indicated that clove oil was effective at 4-fold lower concentrations than 2-phenoxyethanol, but the recovery time was longer than 2-phenoxyethanol. These results suggest that clove oil and 2-phenoxyethanol were effective anesthetics and could be used as anesthetic agents in doctor fish.

Key words: anesthetic agent; anesthesia; induction time; recovery time; essential oil; *Eugenia caryophyllus.*

EFICÁCIA ANESTÉSICA DO PETRÓLEO E DO 2-FENOXIETANOL NO PEIXE DO DOUTOR, *Garra rufa* (HECKEL, 1843)

RESUMO

Este estudo teve como objetivo determinar a eficácia anestésica do óleo de cravo e do 2-fenoxietanol em peixes medicinais (*Garra rufa*) em duas diferentes temperaturas da água. Os peixes (1,2 ± 0,2 g de peso médio) foram expostos a 25, 50, 75 e 100 µL L^{-1} de óleo de cravo e 100, 200, 300, 400 e 500 µL L-1 de 2-fenoxietanol a 15 e 25 ° C. temperatura da água e os tempos de indução e recuperação foram investigados. Os resultados mostraram que os tempos de indução e recuperação nos peixes medicinais foram significativamente afetados pelas concentrações de óleo de cravo e 2-fenoxietanol, bem como pela temperatura da água. A interação de concentração e temperatura da água em todos os tempos de estágio de indução foi significativa no óleo de cravo. Concentração de efeito interativa significativa e temperatura no tempo de recuperação foram encontradas para cada agente anestésico. O tempo de indução diminuiu significativamente com o aumento da concentração de ambos os agentes anestésicos a 15 e 25 ° C da temperatura da água. As menores concentrações efetivas que produziram indução dentro de 3 min e recuperação dentro de 5 min foram 50-75 µL L^{-1} de óleo de cravo e 300 µL L^{-1} de 2-fenoxietanol em 15 e 25 ° C respectivamente para peixes medicinais. Os resultados também indicaram que o óleo de cravo-da-índia era eficaz em concentrações 4 vezes menores do que o 2-fenoxietanol, mas a recuperação foi maior do que o 2-fenoxietanol. Estes resultados sugerem que o óleo de cravo e o 2-fenoxietanol eram anestésicos eficazes e poderiam ser usados como agentes anestésicos em peixes medicinais.

Palavras-chave: anestésico; anesthesia; tempo de indução; tempo de recuperação; óleo essencial; *Eugenia caryophyllus.*

INTRODUCTION

Garra rufa (Doctor fish) is a subtropical freshwater fish species belonging to the *Cyprinidae* and they prefer between 15 and 28 °C water temperature under natural conditions (Baensch and Riehl, 1991). In the last decade, *G. rufa* is getting more popular and commonly used for fish SPA and fish pedicure in the worldwide. These species have been used in ichthyotherapy for alternative treatment of healing of skin diseases such as psoriasis and eczema (Ozcelik et al., 2000; Yedier et al., 2016) so these fish are called "doctor fish". Also, doctor fish has been used in aquarium fish sector due to its feeding strategy which cleans the aquarium (Vazirzadeh et al., 2014). The demand for this fish is increasing day by day in both health tourism and aquaculture sector. The increase in demand for doctor fish subsequently increases the pressure on natural fish stocks. Doctor fish culture is very important for conservation of natural stocks in terms of sustainable tourism and aquaculture. Furthermore, culture of this fish has become a significant economic gain worldwide. It is emphasized that during the aquaculture activities, the use of anesthetic agents is required to maximize fish welfare during handling process (Barata et al., 2016).

Anesthetic agents, both synthetic and plant originated are used in aquaculture procedures to minimize fish activity and to avoid stress and physical damages caused by handling (Priborsky and Velisek, 2018). A good anesthetic agent for fish should induce anesthesia even at low concentrations in less than 3 min and allow recovery within 5 min, should also be cheap and easy to use (Marking and Meyer, 1985; Kizak et al., 2018). The major synthetic anesthetics used in aquaculture are 2-phenoxyethanol (Priborsky and Velisek, 2018), tricaine methanesulphonate (MS-222) and metomidate (Weber et al., 2009), benzocaine (Gökçek et al., 2016), etomidate (Rożyński et al., 2018), propofol and quinaldine sulphate (Priborsky and Velisek, 2018), and ketamine hydrochloride (Adel et al., 2016). Some plant originated essential oils such as basil and lemongrass (Limma-Netto et al., 2016), camphor (Pedrazzani and Neto, 2016), spearmint and lavender (Metin et al., 2015), *Myrcia sylvatica* and *Curcuma longa* (Saccol et al., 2017), *Aloysia triphylla* (Batista et al., 2018), *Lippia alba* (Souza et al., 2018) rosewood (Kizak et al., 2018), geranium (Can et al., 2018), and clove (Javahery et al., 2012; Cunha et al., 2015; Fujimoto et al., 2018; Mitjana et al., 2018) have recently been studied as potential anesthetic agents in aquaculture.

Clove oil as most popular plant originated essential oil as an anesthetic agent is obtained by the distillation of the leaves, stems, and flowers of *Eugenia aromatica* or *Eugenia caryophylata* trees and its active ingredient is eugenol at concentrations of approximately 70-90% by volume (Mylonas et al., 2005; Ross and Ross, 2008; Javahery et al., 2012; Mitjana et al., 2014). Another most widely used anesthetic in aquaculture is 2-phenoxyethanol, which is an aromatic liquid and colorless, and reasonably water-soluble chemical (Hekimoğlu et al., 2017; Mitjana et al., 2018). Clove oil and 2-phenoxyethanol are increasingly used in aquaculture operations due to its low cost, availability, efficacy and easy preparation features in most fish species (Ghanawi et al., 2013; Santos et al., 2015; Adel et al., 2016; Mitjana et al., 2018). Clove oil, also used as a natural anesthetic drug, does not require any withdrawal period in contrast to some anesthetics like MS-222, and it also has been shown to be safe for humans (Javahery et al., 2012). These two anesthetic agents have been evaluated in various fish species such as *Sparus aurata* and *Oncorhynchus mykiss* (Tort et al., 2002), *Dicentrarchus labrax* and *S. aurata* (Mylonas et al., 2005), *Solea senegalensis* (Weber et al., 2009), *Pterophyllum scalare* (Mitjana et al., 2014), *Argyrosomus regius* (Cárdenas et al., 2016), *Acipenser persicus* (Adel et al., 2016), *Silurus glanis* (Gökçek et al., 2016), *Amphiprion ocellaris* and *Xiphophorus helleri* (Hekimoğlu et al., 2017), *Poecilia reticulat*a (Mitjana et al., 2018).

The effective concentrations of anesthetics are depending on the fish species and anesthetic agent (Zahl et al., 2009; Skår et al., 2017). Temperature, pH, age, size, sex, and interactions among these factors also affect the efficacy of anesthetics in fish (Ross and Ross, 2008; Zahl et al., 2009; Mitjana et al., 2018). It is known that the responses of fish to anesthetics can considerably vary between different water temperatures (Akbulut et al., 2012; Santos et al., 2015; Skår et al., 2017). For this reason, it is very important to determine effective anesthetic concentrations for each fish species at different water temperatures. To the best of our knowledge, there is no study on the effects of anesthetic agents on doctor fish. Therefore, the present study aimed to investigate the anesthetic effects of clove oil and 2-phenoxyethanol in doctor fish, and determine their effective anesthetic concentrations. In addition, the effects of interaction between anesthetic concentration and water temperature on the efficacy of the anesthetics were investigated.

MATERIALS AND METHODS

Anesthetic agents

Clove flower (*Eugenia caryophyllus*) essential oil (99% purity, Talya Bitkisel Urünler Ind. Co. Ltd., Antalya, Turkey) and 2-phenoxyethanol (ethylene glycol monophenyl ether, Sigma-Aldrich Inc.) were used as anesthetic agents. According to producer declaration, clove essential oil consists of 80.56% eugenol, 9.77% eugenyl acetate, 7.26% β-caryophyllene and 2.41% other minor constituents.

Fish and experimental conditions

After all experimental protocols' approval of the Akdeniz University Animal Experiments Local Ethics Committee (Date: 24.01.2018, Decision no: 16), the experiment was carried out in April-June 2018 at the Experimental Fish Unit of Fisheries Faculty, Akdeniz University (Antalya, Turkey). Prior to the experiments, a total of 200 doctor fish (*Garra rufa* Heckel, 1843) (1.29 ± 0.24 g mean body weight) were randomly divided into 2 groups (100 fish each group) and put into 2 circular fiberglass tanks (200 L) equipped with continuous aeration and external filter. Two different water temperatures (15 and 25 °C) were applied with the following water quality parameters: pH 7.40 ± 0.11; dissolved oxygen 8.87 ± 0.51 mg L^{-1}; and total ammonia 0.99 ± 0.22 mg L^{-1}. Water temperature of recirculating tank systems were adjusted to 15 and

25 °C with chiller-heater devices (2500 kcal hour^{-1}, Akuakare Products, Mugla, Turkey). Ten percent of water in the tanks was renewed daily by dechlorinated tap water. The photoperiod was provided under a 12 h light:12 h dark cycle by fluorescent lamps. Experimental fish were fed two times a day (9:00 a.m. and 5:00 p.m.) with commercial feed containing 41.0% crude protein, 7.0% crude fat (ArtAkua, İzmir, Turkey) and was allowed for acclimation for 21 days before the anesthetic efficiency experiments.

Anesthetic efficacy experiment

After acclimation, anesthetic efficacy of clove oil and 2-phenoxyethanol were investigated on doctor fish at two different water temperatures. Stock solutions of anesthetic agents were prepared before the experiment as follows: clove oil and 2-phenoxyethanol, each of them was mixed with nine volumes of 95% ethanol to increase water solubility (Yildiz et al., 2013). Induction process was conducted in a 5 L glass container (3 L of water) equipped with aeration. The fish were anesthetized with 25, 50, 75 and 100 µL L^{-1} clove oil and 100, 200, 300, 400 and 500 µL L^{-1} 2-phenoxyethanol concentrations at two different water temperatures (15 and 25 °C) and induction and recovery time were recorded. Ten fish were exposed to each anesthetic concentration for determination of induction time. Each fish was individually caught and placed into the anesthetic container, and used only once. The times required to reach the desired stage of anesthesia (induction time) were recorded based on the fish behavioral responses. The concentration was considered as insufficient for both anesthetic agents when their concentrations did not cause any induction within 15 minutes. The different stages of induction and recovery of anesthesia were determined according to a protocol adapted from Keene et al. (1998) and Cunha et al. (2015) (Table 1). After the induction period, recovery times were evaluated. The fish were removed from anesthesia container and transferred into a 10 L glass container containing 5 L of anesthetic-free water which was supplied from 15 or 25 °C temperature groups' tanks with constant aeration. It was considered as recovered when the fish regained equilibrium and started to swim in the container. After recovery, each fish was transferred into the aquarium to check mortality for 48 h.

Statistical analysis

At first, normality of the data was assessed using a Shapiro-Wilk test and homogeneity of variance was verified using the Levene test. Significant differences among means were compared using ANOVA, followed by the Bonferroni's post hoc test. Two-way analysis of variance (ANOVA) was used to test for the significance of the effects of anesthetic agent concentrations, water temperatures, and its interaction (concentration x water temperature). The relationship between each of the stages of anesthesia and anesthetic agent concentration was examined using regression analysis (concentration × time of anesthesia induction; concentration × time of recovery from anesthesia). Statistical analyses were conducted using the SPSS software (v23, IBM Corporation, New York, USA). The results are presented as means ± SD and differences were considered statistically significant when $P<0.05$.

RESULTS

At the end of the experimental anesthetic administration, no mortality was detected 48 h after exposure to clove oil and 2-phenoxyethanol concentrations. The induction time for anesthesia stage 1, stage 2, stage 3 and recovery time for doctor fish at two water temperatures (15 and 25 °C) are given in Table 2. 100 µL L^{-1} 2-phenoxyethanol concentration was not sufficient to induction (stage 3) to anesthesia in both water temperatures within 15 min. Clove oil was found to be anesthetic at all concentrations (25-100 µL L^{-1}) and 2-phenoxyethanol was found to be anesthetic at 200 µL L^{-1} and above concentrations (200-500 µL L^{-1}). However, 25 µL L^{-1} of clove oil at 15 °C water temperature was not sufficient to reach to stage 3 within 10 min. The shortest time to induction (stage 3) time were 96.2 sec in 15 °C and 68.1 sec in 25 °C at 100 µL L^{-1} for the clove oil. For the shortest induction (stage 3) times for 2-phenoxyethanol at the concentration of 500 µL L^{-1} was 63.4 sec in 15 °C water temperature and 54.0 sec in 25 °C water temperature. The different anesthetic agents resulted in different induction and recovery times. Induction times decreased when two anesthetic agent concentrations increased at 15 °C water temperature (Figure 1). A similar relationship was obtained at 25 °C as well (Figure 1). Whereas, higher anesthetic agent concentrations caused prolongation on recovery time significantly for two anesthetics at both water temperatures (Figure 2). In higher

Table 1. Behavioral observations of anesthesia stages.

Stages	Exhibited behavior
Induction	
Stage 1	Relaxation and no response to stimuli: fish calm and do not respond to tactile touch, but respond to external stimuli (a blow on the anesthetic chamber); opercular rate increases.
Stage 2	Imbalance swimming: fish loss their equilibrium and show imbalance swimming; normal opercular rate; response to external stimuli.
Stage 3	Total loss of equilibrium and movement: fish lay on lateral side; no movement; no response to external stimuli; slightly depressed opercular rate.
Recovery	Total behavioural recovery. Fish began to normal swimming behavior in the container.

Behavioral observations of anesthesia stages adapted from Keene et al. (1998) and Cunha et al. (2015).

Table 2. Induction and recovery time (second) of different concentrations of clove oil and 2-phenoxyethanol on doctor fish at different water temperatures.

Concentrations ($\mu L\ L^{-1}$)	15 °C water temperature				25 °C water temperature			
	Stage 1	Stage 2	Stage 3	Recovery	Stage 1	Stage 2	Stage 3	Recovery
Clove oil								
25	65.8 ± 7.9^{a}	139.4 ± 9.6^{a}	764.4 ± 23.8^{a}	231.3 ± 28.6^{d}	36.5 ± 7.6^{a}	71.8 ± 8.8^{a}	231.4 ± 31.2^{a}	268.7 ± 21.1^{d}
50	34.2 ± 6.4^{b}	62.1 ± 5.8^{b}	225.6 ± 33.9^{b}	276.8 ± 22.8^{c}	24.6 ± 4.2^{b}	44.2 ± 5.3^{b}	167.7 ± 20.4^{b}	317.5 ± 21.8^{bc}
75	27.6 ± 4.1^{c}	48.2 ± 3.4^{bc}	162.9 ± 22.8^{c}	322.6 ± 36.9^{b}	19.7 ± 2.4^{bc}	32.3 ± 10.4^{bc}	89.5 ± 13.3^{bc}	345.0 ± 45.7^{ab}
100	20.6 ± 3.0^{d}	39.8 ± 3.9^{c}	96.2 ± 13.2^{d}	392.5 ± 20.4^{a}	16.4 ± 2.1^{c}	25.5 ± 5.5^{c}	68.1 ± 10.1^{c}	372.5 ± 19.1^{a}
2-phenoxyethanol								
100	115.6 ± 14.4^{a}	401.4 ± 32.2^{a}	-*	-	107.1 ± 11.6^{a}	382.0 ± 26.2^{a}	-*	-
200	71.6 ± 8.8^{b}	109.5 ± 15.6^{b}	236.2 ± 33.6^{a}	181.1 ± 13.7^{ab}	64.1 ± 7.2^{b}	86.2 ± 6.5^{b}	207.8 ± 18.6^{a}	111.5 ± 16.8^{c}
300	42.1 ± 7.6^{cd}	57.7 ± 7.9^{c}	94.2 ± 14.2^{b}	174.6 ± 12.7^{bc}	36.7 ± 5.2^{c}	51.4 ± 7.7^{c}	80.3 ± 10.0^{b}	119.2 ± 19.9^{bc}
400	34.6 ± 5.6^{d}	42.9 ± 5.5^{cd}	71.7 ± 6.1^{c}	183.3 ± 12.5^{ab}	25.7 ± 5.8^{de}	35.4 ± 5.3^{cd}	60.5 ± 7.6^{c}	134.1 ± 14.6^{b}
500	19.5 ± 3.5^{e}	34.0 ± 5.9^{de}	63.4 ± 5.6^{cd}	196.6 ± 15.9^{a}	20.2 ± 3.7^{e}	31.3 ± 3.9^{de}	54.0 ± 7.2^{cd}	168.1 ± 20.8^{a}

*Anesthetic concentration was not sufficient to induction to anesthesia within 15 minutes. Data are expressed as Mean ± SD (N=10). Values with different superscripts in each column are significantly different (P<0.05).

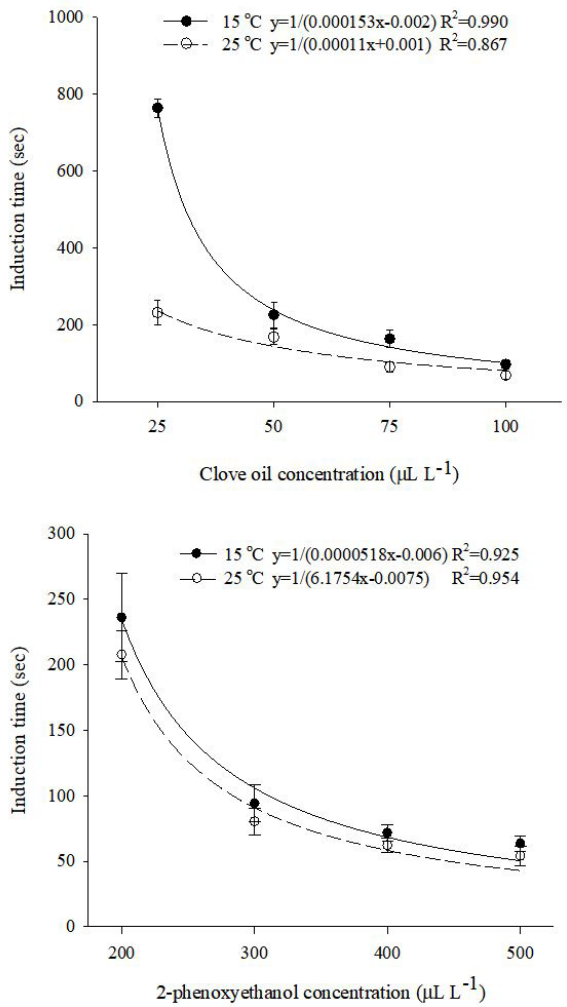

Figure 1. Relationships between induction time (Stage 3) of doctor fish in different water temperatures exposed to different concentrations of clove oil and 2-phenoxyethanol. Mean ± SD (N=10).

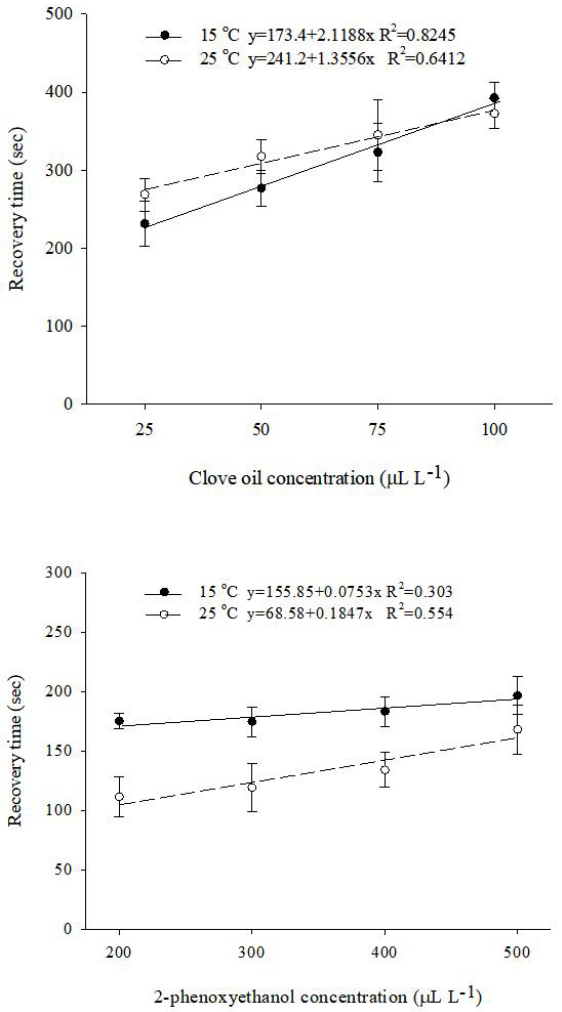

Figure 2. Relationships between recovery time in doctor fish at different water temperatures exposed to different concentrations of clove oil and 2-phenoxyethanol. Mean ± SD (N=10).

water temperature (25 °C) caused shorter recovery times in all 2-phenoxyethanol concentrations, apart from fish anesthetized with 75-100 μL L^{-1} concentrations of clove oil. Clove oil and 2-phenoxyethanol concentrations significantly affected all anesthetic induction stages (Stage 1, stage 2 and stage 3) and recovery time at two water temperatures (Table 2). Similarly, water temperature significantly affected the anesthetic induction and recovery times for both anesthetic agents. Two-way ANOVA revealed not only anesthetic agent concentrations but also water temperatures played a significant role on anesthesia of doctor fish ($P<0.001$). Furthermore, the interaction of water temperatures and anesthetic concentrations on all induction and recovery times for clove oil, and recovery times for 2-phenoxyethanol was also significant ($P<0.001$).

DISCUSSION

There are several studies which try to determine the effective concentrations of clove oil and 2-phenoxyethanol in *S. aurata* and *D. labrax* (Mylonas et al., 2005), *S. senegalensis* (Weber et al., 2009), *A. persicus* (Adel et al., 2016), *A. regius* (Barata et al., 2016). However, there is no anesthetic efficacy study in doctor fish regarding these anesthetic drugs. According to Marking and Meyer (1985), an ideal anesthetic agent for fish should induce anesthesia in less than 3 min and allow recovery in 5 min. Our study demonstrated that the effective concentrations in 15 and 25 °C water temperature that produced induction time (Stage 3) within 3 min and recovery time within 5 min were 50-75 μL L^{-1} for clove oil and 300 μL L^{-1} for 2-phenoxyethanol, respectively (Table 2, Figure 1). Similar (Mylonas et al., 2005; Serezli et al., 2012; Adel et al., 2016) and different results have been observed by other researchers in *S. senegalensis* (Weber et al., 2009), *Huso huso* (Shaluei et al., 2012), *P. scalare* (Mitjana et al., 2014), *P. reticulata* (Cunha et al., 2015). The effective clove oil and 2-phenoxyethanol concentrations to induce anesthesia in fish species varies between 27-100 μL L^{-1} and 200-1400 μL L^{-1}, respectively (Javahery et al., 2012; Pedrazzani and Neto, 2016; Fujimoto et al., 2018; Mitjana et al., 2018). Effective concentrations of anesthetic agents in the present study seem to be among these values. The ratio of the major components (Eugenol: 80.56%) in the content of the clove oil used in the present study seems to be compatible with the eugenol ratios approximately 70-90% in other studies (Akbulut et al., 2012; Ghanawi et al., 2013). Hekimoğlu et al. (2017) stated eugenol ratio in clove oil was 96.1%. As in Hekimoğlu et al. (2017), the ratio of eugenol in clove oil could be different from this range in some studies, and this may cause variations in the results. As reported by Mylonas et al. (2005), we found that clove oil was effective at 4-fold lower concentrations than 2-phenoxyethanol on doctor fish. Barata et al. (2016) explained this situation that clove oil affects different type of receptors might justify a higher efficiency and the use of lower concentrations. It is clear that the clove oil is advantageous because of its plant origin, safe nature, low price, and its effectiveness even at lower concentrations (Mylonas et al., 2005; Javahery et al., 2012; Kizak et al., 2018). In agreement with previous findings for *H. huso* (Shaluei et al., 2012), concentration of 100 μL L^{-1} 2-phenoxyethanol was inadequate for induce anesthesia in doctor fish. It is possible that 100 μL L^{-1} of 2-phenoxyethanol concentrations (or lower than that) can be used to induce sedation during transportation or other procedures.

Induction times in both water temperature decreased significantly with increasing concentrations of clove oil or 2-phenoxyethanol ($P<0.05$). There was a strong negative relationship between clove oil concentration and induction time at water temperature of 15 °C ($R^2 = 0.990$) and 25 °C ($R^2 = 0.867$) (Figure 1). The relationship was also recorded between induction time and 2-phenoxyethanol concentration at water temperature of 15 °C ($R^2 = 0.925$) and at 25 °C ($R^2 = 0.954$) (Figure 1). Similarly, negative relationship between anesthetic agent concentrations and induction time of anesthesia were reported in *D. labrax* and *S. aurata* (Mylonas et al., 2005), *S. senegalensis* (Weber et al., 2009), *Acipenser gueldenstaedtii* (Akbulut et al., 2011), *Siganus rivulatus* (Ghanawi et al., 2013), *P. scalare* (Mitjana et al., 2014), *A. persicus* (Adel et al., 2016), *S. glanis* (Gökçek et al., 2016), *Colossoma macropomum* (Saccol et al., 2017), *Carassius auratus* (Kizak et al., 2018).

In this study, it was determined that the recovery time at both water temperatures was shorter in the 2-phenoxyethanol concentrations than clove oil, which is an important criterion in selecting anesthetic agents. Similar result has been demonstrated in *D. labrax* (Mylonas et al., 2005). While recovery time at 25 °C water temperature was less than 5 min in all 2-phenoxyethanol concentrations in the present study, the time was over 5 min at clove oil concentrations except 25 μL L^{-1}. Higher anesthetic concentrations in clove oil and 2-phenoxyethanol resulted in higher recovery times in both water temperatures in the present study ($P<0.05$) (Figure 2). We found a positive relationship between anesthetic concentration and recovery time in this study. Similar effects have been demonstrated in *A. gueldenstaedtii* (Akbulut et al., 2011), *A. persicus* (Adel et al., 2016), *S. glanis* (Gökçek et al., 2016) and *C. auratus* (Kizak et al., 2018). However, it was reported that similar or shorter recovery time in *P. scalare* (Mitjana et al., 2014), *S. senegalensis* (Weber et al., 2009), *S. rivulatus* (Ghanawi et al., 2013) exposed to clove oil, and *C. macropomum* (Saccol et al., 2017) exposed to *M. sylvatica* and *C. longa* essential oils. Also, Mirghaed et al. (2016) and Bolasina et al. (2017) could not find positive relationships between anesthetic concentration and recovery time. Weber et al. (2009) and Mitjana et al. (2014) explained that differences among the studies might be clarified if the specific properties of each species is taken into account, such as the physiological responses of fish to anesthetic agents. In addition, it is stated that the pharmacokinetics of the anesthetic agent may cause differences among studies (Zahl et al., 2009; Bolasina et al., 2017).

Anesthetic concentration and various biological or environmental factors significantly affect the anesthesia of fish (Santos et al., 2015; Li et al., 2018; Mitjana et al., 2018). Changes in water temperature have been shown to affect induction and recovery time in various fish species (Hamackova et al., 2004; Mylonas et al., 2005; Zahl et al., 2009; Santos et al., 2015; Skår et al., 2017). In the present experiments, 15 and 25 °C water temperatures were preferred on doctor fish anesthesia because this cyprinid fish is

generally found in the natural habitats at water temperatures of 15-25 °C (Vazirzadeh et al., 2014). Our results showed that a rise in water temperature from 15 to 25 °C shortened both induction and recovery times for 2-phenoxyethanol, and induction time for clove oil. For example, the induction time to stage 3 anesthesia for clove oil concentrations varied from 764 s to 96 s at water temperature of 15 °C, and from 231 s to 68 s at 25 °C. Water temperature significantly affects stage 1, stage 2, stage 3, and recovery time in both clove oil and 2-phenoxyethanol ($P<0.05$) (Table 2). Furthermore, statistically significant interactions were detected between water temperature and anesthetic concentration ($P<0.001$). A negative interaction between water temperature and anesthetic concentration was clearly seen in almost all the results. Similar findings have been reported for clove oil by Hoskonen and Pirhonen (2004). Shortened induction and recovery times with increasing water temperature have also been shown for clove oil or 2-phenoxyethanol in *Tinca tinca* (Hamackova et al., 2004), *S. aurata* and *D. labrax* (Mylonas et al., 2005), *Epinephelus bruneus* (Park et al., 2008), *Gadus morhua* (Zahl et al., 2009), *O. mykiss* (Yildiz et al., 2013) and *Siganus rivulatus* (Santos et al., 2015). Reduced induction and recovery times with increased temperatures from 6 to 12 °C have been reported by Skår et al. (2017) for *Cyclopterus lumpus* anesthetized with metacaine, benzocaine, and isoeugenol. The interactive effects of water temperature and anesthetic concentration have been well documented in this study and literature. This reduction in time of induction and recovery of anesthesia as water temperature increases is possibly related to the acceleration of the opercular ventilation rate and gill blood flow owing to the increased basal metabolism of fish maintained at higher water temperatures (Zahl et al., 2009; Silva et al., 2012; Santos et al., 2015; Skår et al., 2017). It is thought that changes in electrocardiographic responses may play a role in obtaining these results due to concentration, temperature, or interaction between these factors. Santos et al. (2015) stated that increasing metabolic rate accelerates respiration, and increases cardiac output and blood flow through the gills. Furthermore, Barbas et al. (2017) reported a depressant effect on cardiac rhythm and decreased heart rates that occurred during *C. macropomum* anesthesia with essential oil of citronella, *Cymbopogon nardus*. From all these results, it is concluded that the change in fish physiology due to the change in water temperature significantly affects the duration of the anesthesia induction and recovery time.

CONCLUSIONS

In conclusion, the results indicate that clove oil and 2-phenoxyethanol can be used as effective anesthetic agents for doctor fish anesthesia. The present study demonstrated that the minimum effective concentration of clove oil was determined as 75 μL L^{-1} at water temperature of 15 °C and 50 μL L^{-1} at 25 °C, and 300 μL L^{-1} at both temperatures for 2-phenoxyethanol. However, further studies are needed to determine the effects of anesthetic agents on doctor fish of different sizes and the physiological responses.

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