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Effects of thymol and carvacrol anesthesia on the electrocardiographic and behavioral responses of the doctor fish *Garra rufa*

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ABSTRACT

The present study examined the anesthetic efficiency and electrocardiographic and behavioral responses of the doctor fish *Garra rufa* anesthetized with thymol and carvacrol. Fish were subjected to anesthetic baths in five different concentrations of the thymol (25, 50, 75, 100, and 150 mg L^{-1}) and carvacrol (25, 50, 75, 100, and 150 μL L^{-1}). Induction and recovery times were recorded for each fish separately, and 10 fish were used per anesthetic concentration. Video tracking and processing were performed for each anesthetic to obtain changes in behavioral parameters between anesthetic concentrations. Thereafter, a different experiment was established for cardiographic evaluations, and necessary electrocardiographic (ECG) records were obtained in 5 fish. At the end of the experiments, all tested thymol and carvacrol concentrations effectively induced anesthesia in *G. rufa*. Video tracking analyses demonstrated that *G. rufa* did not exhibit any changes in behavioral parameters when submitted to 25–50 mg L^{-1} concentrations of thymol compared to anesthetic-free water. However, movement distance and mobility significantly increased with increasing carvacrol concentrations. We found that high concentrations of thymol (150 mg L^{-1}) and carvacrol (100 and 150 μL L^{-1}) caused avoidance behaviors in *G. rufa*. ECG recordings showed that heart rate decreased, and PR and QT intervals were prolonged with increased thymol concentrations. Thymol concentrations did not affect PQ intervals. No significant changes in ECG parameters were observed with carvacrol. These results demonstrated that concentrations of 25 and 50 mg L^{-1} thymol and 50 μL L^{-1} carvacrol can be used for general aquaculture procedures, and 100 mg L^{-1} thymol and 75 μL L^{-1} carvacrol can be used as rapid anesthesia in *G. rufa*.

1. Introduction

Garra rufa is a freshwater fish species belonging to the *Cyprinidae* family that is naturally distributed in Turkey (Aydın and Akhan, 2020; Yedier et al., 2016). This fish is an important aquaculture species, and it was mostly evaluated for use in fish spas and fish pedicures in Turkey and most other countries (Aydın et al., 2019). In health tourism, these fish are called “doctor fish” due to its use as an alternative treatment in the healing of skin diseases, such as psoriasis and eczema (Aydın and Akhan, 2020; Ozcelik et al., 2000; Yedier et al., 2016). The demand for this fish increases daily. Therefore, the production of *G. rufa* fish is very important for the sustainability of natural fish stocks and the tourism industry. Anesthetic agents are commonly used during aquaculture procedures to minimize fish stress and maximize fish welfare during the handling process (Aydın et al., 2019; Barbas et al., 2020; Cunha et al., 2010).

The use of plant-based substances which can be used as an alternative to synthetic substances in various fields and have less negative impact on human health and the environment, increased in recent years. The reason for focusing on plant-based substances is that these substances are already present in nature and less detrimental effect for soil and water (Mylonas et al., 2005). Plant-based anesthetics are inexpensive to manufacture and abundant (Aydın and Barbas, 2020). Some of the plant-based anesthetics that are used for fish anesthesia have antifungal, antiparasitic, antibacterial and antiviral properties (Teta and Kaiser, 2019). Therefore, several recent trials investigated the sedative and anesthetic properties of essential oils and their active substances in fish species (Aydın and Barbas, 2020; Boaventura et al., 2020; Can et al., 2018, 2019; Cunha et al., 2010; de Freitas Souza et al., 2018; Gökçek et al., 2017; Hoseini et al., 2019). Some recent studies showed that plant-based active substances, such as 1,8-cineole (Hoseini et al., 2020; Mazandarani and Hoseini, 2017; Taheri Mirghaed et al., 2018a),

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eugenol (Taheri Mirghaed et al., 2018b; Tarkhani et al., 2017a), menthol (Mazandarani and Hoseini, 2017; Pereira-da-Silva et al., 2016; Teta and Kaiser, 2019; Zapata-Guerra et al., 2020), myrcene (Taheri Mirghaed et al., 2018b, 2016), linalool (Mazandarani et al., 2017; Silva et al., 2017; Yousefi et al., 2019), citronellal (Yousefi et al., 2019; Taheri Mirghaed et al., 2018b), propofol (de Souza et al., 2019; Mitjana et al., 2018) and 4-allylphenyl acetate (Khumpirapang et al., 2018) were investigated for anesthesia in various fish species (Aydın and Barbas, 2020). Thymol (*Thymus spp*.) and carvacrol (*Origanum spp*.), which are the main compounds of the thyme and oregano essential oils, have anesthetic effects, but relatively few studies evaluated the effects of these anesthetics on fish (Bianchini et al., 2017; Yousefi et al., 2018a).

With the development of technology, various initiatives have been used and developed to support research and farming. One of these technological advances is the implementation of video tracking technology and tracking software program in aquaculture research (Delcourt et al., 2013; Noldus et al., 2001). Some studies documented in the literature have focused on behavioral analysis of aquatic species (Boyle et al., 2013; Delcourt et al., 2013; Jijie et al., 2020; Simão et al., 2019; Stewart et al., 2015; Strungaru et al., 2019; Vera et al., 2010). However, few studies have examined the effects of anesthesia on fish swimming behavior. Therefore, the present study investigated the effects of two anesthetics on movement distance, swimming speed and mobility of *G. rufa*. Because these behavioral parameters is a good indicator to evaluate stress level and welfare in fish (Ashley, 2007; Martins et al., 2012).

Hematological and biochemical parameters are frequently examined in anesthesia studies, but detailed study of behavioral and cardiological evaluations is lacking in the literature (Aydın and Barbas, 2020). Few studies recently evaluated the electrocardiograms (ECG) of fish (Barbas et al., 2017; de Souza et al., 2019; Hill and Forster, 2004; Huang et al., 2010; Ma et al., 2019). ECG, which consists of recording the electrical activity of the heart muscle can be used in the diagnosis of heart health when fish exposed the anesthetics (de Souza et al., 2019). de Souza et al. (2019) reported cardiac function was transiently affected, with decreased heart beats under exposure to propofol and essential oil of *Nepeta cataria*. It is also reported significant reductions in heart and ventilation rates of Arctic charr (*Salvelinus alpinus*) during exposure to the carbon dioxide (CO_2) treated water (Seth et al., 2013). Objective monitoring is necessary for full assessment of the condition of fish exposed the anesthetics. Therefore, electrocardiographic recordings of fish in anesthesia studies might enable a more accurate assessment of the anesthetic efficacy.

The objective of the present study was to investigate the effects of thymol and carvacrol on induction and recovery times of fish and investigated electrocardiographic parameters. Furthermore, the effects of the anesthetics on behavioral parameters such as movement distance, swimming speed and mobility were also evaluated.

2. Materials and methods

2.1. Fish and maintenance

A total number of 140 doctor fish *Garra rufa* (1.8 ± 0.2 g average body weight) were used in this anesthesia study. The fish were stocked in 250-L circular fiberglass tanks with mechanical and biological filters. Tanks were continuously aerated, and the daily water exchange rate was 5%. Water quality parameters of the stock tanks and experimental plastic containers were measured and maintained at pH = 7.5 ± 0.2, temperature = 25.0 ± 1.0 °C, dissolved oxygen = 8.1 ± 0.3 mg L^{-1} and total ammonia nitrogen (TAN) ($NH_4^+ + NH_3^- - N$) = 0.09 ± 0.02 mg L^{-1}. A constant photoperiod set at 12:00 h light: 12:00 h darkness under fluorescent lighting. Prior to experiments, the fish were hand-fed twice (09.00 a.m. and 16.00 p.m.) daily until apparent satiation using commercial feed (41% crude protein, 7% crude lipid) for 21 days for acclimatization. Feeding stopped 24 h before the experiments.

2.2. Anesthetic agents and preparation

Thymol (99% purity; Sigma-Aldrich Corporation, St. Louis, MO, USA) and carvacrol (98% purity; Sigma-Aldrich Corporation, St. Louis, MO, USA) were purchased from commercial sources as anesthetic agents. The anesthetics were dissolved in 95% ethanol (Tekkim Kimya, Bursa, Turkey) at a ratio of 1:10 to increase water solubility, and the mixture was prepared just before the anesthetic experiments (Kizak et al., 2018).

2.3. Experiment 1: Anesthetic efficiency and behavioral analysis

A total of 110 fish were used to assess induction and recovery times for thymol and carvacrol anesthesia and behavioral analysis. The fish were stocked for 3 h in 11 continuously aerated plastic containers ($40 \times 40 \times 40$ cm; length*width*height) (5 L of water) in equal numbers and allowed to acclimatize to the experimental conditions. The fish were exposed to five different thymol (25, 50, 75, 100, and 150 mg L^{-1}) and carvacrol (25, 50, 75, 100, and 150 μL L^{-1}) concentrations. Each fish ($n = 10$ each anesthetic concentration) was individually transferred into a continuously aerated plastic container ($30 \times 30 \times 30$ cm; length*-width*height) (3 L of water) containing the test anesthetic concentration in water. The anesthesia and recovery stages observed in the experiment were detailed by Aydın et al. (2019) and Can and Sümer (2019). The induction times were recorded during stage 1, which is characterized by the loss of reactivity to external stimuli, except strong pressure, and stage 2, which is a total loss of equilibrium and no response to external stimuli. The times required to reach the desired stage of anesthesia (induction time) were recorded based on the behavioral responses. During the induction stages, video recordings were taken to evaluate changes in behavior of the 5 fish for each concentration of thymol and carvacrol. The fish were transferred to anesthetic-free and continuously aerated plastic containers ($40 \times 40 \times 40$ cm; length*width*height) (5 L of water) to evaluate the recovery time from the test anesthetic concentration. The fish were considered recovery stage 1 when a partial recovery of equilibrium with partial recovery of swimming motion was observed. The fish were considered recovery stage 2 when normal swimming returned.

A video recording system was used to assess behavioral responses to anesthetic exposure. The video recording system consisted of a continuously aerated white container ($30 \times 30 \times 30$ cm; length*width*height, and water depth: 10 cm) (3 L of water with anesthetic concentrations) on which a digital camera was placed 45 cm from the bottom of the container for monitoring, and a bottom light unit (30-cm round LED panel light, 18 W, 5.000 K) was used to illuminate the arenas. The movement of fish during induction was recorded using a digital camera for 1 min (Fig. 1). This video recording application was performed for each anesthetic concentration under similar conditions. Fish ($n = 10$) behavior in anesthetic-free water (0 μL L^{-1} anesthetic agent) was also recorded. The recorded video files were subsequently analyzed using Ethovision™ XT 14 software (Noldus Information Technology, Wageningen, Netherlands). The following parameters were analyzed: movement distance (cm) (Distance travelled by fish during the recording), swimming speed (velocity, cm s^{-1}) (Swimming speed of the fish during the recording) and mobility (%) (Movement or the change in position of the fish). After the experiments, survival was monitored up to 48 h.

2.4. Experiment 2: Electrocardiogram (ECG) recording

For the electrocardiogram recordings in fish, the methodology of Barbas et al. (2017) and Cantanhêde et al. (2020) was used. Briefly, ECG recording was performed in a specially designed ECG recording chamber to avoid interference caused by electromagnetic radiation. After the fish were anesthetized with the anesthetic concentrations, positive and negative electrodes were fixed into the pericardial cavity from the ventral side of the fish at a depth of approximately 1 mm, and the

Fig. 1. Top view image of the experimental fish in the video tracking system. Detection and marking of the tracking fish.

grounding electrode was inserted approximately 1 mm between the pelvic fins as shown in Fig. 2A. The exact position of the electrodes was adjusted to obtain the maximum voltage signals. The ECG signals were amplified and translated using a high impedance amplifier (ML846 Power Lab system; AD Instruments, Oxford, United Kingdom), analyzed using LabChart 8 software (AD Instruments, Oxford, United Kingdom), filtered at a bandwidth of 0.5 Hz to 200 Hz and digitalized at 50 Hz. The ECG signals were obtained over a 90-s period and 5 fish individually were used to record ECG data in each thymol (50, 100, and 150 mg L^{-1}) and carvacrol (50, 100, and 150 μL L^{-1}) concentration. Heart rate (HR; beats per min) was determined via measurement of the interval between R waves for one min (Fig. 2B), and ECG intervals (PQ interval, PR interval and QT interval) were determined as shown and explained in Fig. 2C. Eight measurements were taken from the ECG record of each fish for electrocardiographic parameters.

2.5. Statistical analyses

All data are presented as the means ± standard deviation (SD). Data normality and homogeneity of the variances were tested and confirmed using Shapiro–Wilk and Levene tests, respectively. One-way ANOVA and Tukey tests were used to find the significant differences among the means. Differences were considered statistically significant when the calculated *P* value was less than 0.05. All analyses were performed in SPSS software (Version 23, IBM Corp., Armonk, New York, USA).

3. Results

3.1. Induction and anesthetic recovery

As seen in Table 1, all of the tested thymol and carvacrol concentrations had anesthetic effects on *G. rufa*. All thymol and carvacrol concentrations were effective in promoting anesthesia and recovery, and no mortality was observed during the anesthesia experiment or 24 h afterwards. The lowest effective anesthetic concentrations of thymol and carvacrol were 25–50 mg L^{-1} (202.2 ± 23.6 s and 129.7 ± 28.3 s, respectively) and 50 μL L^{-1} (145.3 ± 20.9 s), respectively. Thymol and carvacrol concentrations of 50–150 mg L^{-1} and 50–150 μL L^{-1}, respectively, resulted in induction times (Stage 2) less than 180 s. Thymol and carvacrol concentrations significantly affected all anesthetic induction and recovery times (Table 1). Induction times (Stage 1 and Stage 2) negatively correlated with thymol and carvacrol concentrations. Recovery times (Stage 2) for thymol and carvacrol ranged from 268.8 ± 33.7 s to 508.2 ± 62.6 s and 234.2 ± 26.3 s to 361.8 ± 65.3 s, respectively. Carvacrol concentrations of 50–150 μL L^{-1} resulted in a recovery time (Stage 2) less than 300 s.

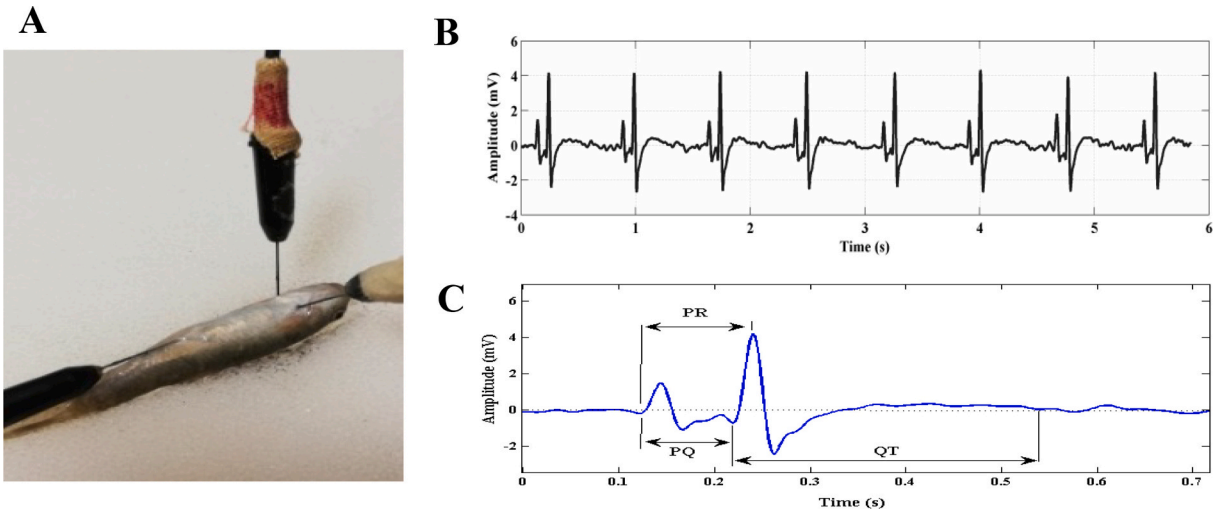

Fig. 2. Electrocardiogram (ECG) electrode placement and measurement of ECG intervals.
A) Electrode connection points at each corner of the fish heart, as indicated by the theoretical triangle; **B)** Illustration of the ECG recordings in *G. rufa* containing various waveforms during anesthetic induction with eugenol; **C)** Standard measurement of ECG intervals (PQ interval: between the P wave onset and QRS onset; PR interval: time from the onset of atrial depolarization to onset of ventricular depolarization; QT interval: time from the onset of Q to the end of T).

Table 1
Induction and recovery times of different concentrations of thymol and carvacrol on *G. rufa*.

	Induction time (s)		Recovery time (s)	
	Stage 1	Stage 2	Stage 1	Stage 2
Thymol concentration (mg L^{-1})				
25	60.7 ± 3.5^{a}	202.2 ± 23.6^{a}	159.7 ± 27.9^{d}	268.8 ± 33.7^{c}
50	45.3 ± 5.9^{b}	129.7 ± 28.3^{b}	273.0 ± 42.8^{c}	427.2 ± 44.1^{b}
75	38.2 ± 3.9^{c}	91.2 ± 12.2^{c}	311.3 ± 39.5^{b}	470.8 ± 73.4^{ab}
100	26.1 ± 3.4^{d}	65.6 ± 5.8^{d}	343.1 ± 25.2^{a}	499.4 ± 52.3^{a}
150	19.8 ± 2.9^{e}	46.9 ± 6.6^{e}	345.9 ± 36.8^{a}	508.2 ± 62.6^{a}
Equations	$y = 0.0022 \times^{2} - 0.7157 \times + 76{,}938$	$y = 0.0126 \times^{2} - 3.4102 \times + 275.79$	$Y = 0.0206 \times^{2} + 5.0106 \times + 56.032$	$y = 0.0271 \times^{2} + 6.4918 \times + 139.43$
	R^2=0.9241	R^2=0.9087	R^2=0.7931	R^2=0.7417
Carvacrol concentration (μL L^{-1})				
25	71.7 ± 9.6^{a}	342.1 ± 77.8^{a}	267.7 ± 42.6^{a}	361.8 ± 65.3^{a}
50	44.0 ± 5.7^{b}	145.3 ± 20.9^{b}	206.0 ± 40.4^{b}	280.6 ± 37.8^{b}
75	34.6 ± 7.4^{c}	84.2 ± 8.7^{c}	172.9 ± 23.0^{b}	234.2 ± 26.3^{b}
100	28.3 ± 6.0^{d}	62.8 ± 6.7^{cd}	183.2 ± 27.3^{b}	244.1 ± 37.8^{b}
150	19.4 ± 3.8^{e}	48.8 ± 6.9^{d}	200.7 ± 40.6^{b}	279.7 ± 60.7^{b}
Equations	$y = -7E\text{-}05 \times^{3} + 0.0216 \times^{2} - 2.3739 \times + 118.32$	$y = -0.0005 \times^{3} + 0.1697 \times^{2} - 18.017 \times + 693.02$	$y = -0.0002 \times^{3} + 0.0598 \times^{2} - 6.4004 \times + 394.05$	$y = -0.0002 \times^{3} + 0.0687 \times^{2} - 7.8603 \times + 519.46$
	R^2= 0.8857	R^2= 0.9048	R^2= 0.4840	R^2= 0.4896

Data are expressed as the means ± SD (n=10).
Values with different superscripts in each column are significantly different (P<0.05).

3.2. The effects of anesthetic concentrations on the behavioral parameters

Several observations related to the swimming behavior of fish for the first 1 min after the release of the fish into the water containing thymol or carvacrol concentrations are given in Table 2. The data showed no significant changes in movement distance and mobility between thymol concentrations (25–150 mg L^{-1}). However, there was a significant difference in mobility between anesthetic-free water (0 μL L^{-1} anesthetic agent) and 100–150 mg L^{-1} thymol. Significant differences were observed between anesthetic-free water and 150 mg L^{-1} thymol concentration in all the behavioral parameters. Significant differences were also detected in all of the behavioral parameters for the carvacrol concentrations. The movement distance of fish anesthetized with thymol and carvacrol ranged from 557.9 ± 61.7 cm to 709.4 ± 95.2 cm and 383.8 ± 111.5 cm to 870.7 ± 105.4 cm, respectively, and increased significantly with increasing carvacrol concentrations (Table 2) (Fig. 3). Swimming speed and mobility also increased significantly with the increase in carvacrol concentrations.

Table 2
Behavioral responses to different concentrations of thymol and carvacrol on *G. rufa*.

	Movement distance (cm)	Swimming speed (Velocity, cm s^{-1})	Mobility (%)
Thymol concentration (mg L^{-1})			
0*	557.95 ± 61.73^{b}	9.94 ± 0.84^{b}	15.47 ± 2.75^{b}
25	627.30 ± 68.14^{ab}	9.51 ± 0.60^{b}	18.69 ± 1.51^{ab}
50	564.88 ± 46.22^{b}	12.33 ± 2.30^{ab}	17.54 ± 1.38^{ab}
75	613.66 ± 66.78^{ab}	12.78 ± 2.21^{ab}	20.35 ± 1.77^{a}
100	693.43 ± 80.10^{ab}	14.01 ± 2.47^{ab}	21.88 ± 3.34^{a}
150	709.43 ± 95.18^{a}	14.50 ± 1.51^{a}	21.71 ± 3.07^{a}
Carvacrol concentration (μL L^{-1})			
0*	519.56 ± 82.34^{bc}	8.91 ± 1.63^{bc}	16.20 ± 0.99^{bc}
25	383.84 ± 111.50^{c}	6.13 ± 2.82^{c}	13.19 ± 0.91^{d}
50	495.48 ± 134.02^{bc}	6.89 ± 3.41^{bc}	13.69 ± 1.10^{cd}
75	564.63 ± 137.35^{bc}	9.12 ± 2.92^{bc}	16.93 ± 1.83^{b}
100	649.35 ± 96.20^{b}	11.49 ± 1.98^{ab}	20.31 ± 1.32^{a}
150	870.72 ± 105.38^{a}	14.69 ± 3.39^{a}	22.63 ± 2.49^{a}

Data are expressed as the means ± SD (n=5). Values with different superscripts in each column are significantly different (P<0.05).
* Anesthetic-free water.

3.3. The effects of anesthetic concentrations on ECG parameters

The effects of thymol and carvacrol concentrations on the electrophysiological cardiac parameters of anesthetized *G. rufa* are given in Table 3. The ECG results indicate that a negative correlation between the thymol concentration and mean heart rate (HR) was observed. However, there was no relationship between carvacrol concentrations and HR. ANOVA revealed that HR were significantly lower in the 150 mg L^{-1} (57.0 ± 7.2 beats min^{-1}) thymol concentration compared to 50 mg L^{-1} (88.2 ± 10.6 beats min^{-1}). The highest reduction in HR was observed at the 150 μL L^{-1} carvacrol concentration, but this reduction was not statistically significant. Table 3 shows that significant differences were detected in PR and QT intervals for the fish induced with thymol concentrations. However, no significant differences were seen in any electrocardiographic parameters for the carvacrol concentrations.

4. Discussion

An ideal anesthetic agent should induce anesthesia in less than 180 s and have an anesthetic recovery time of less than 300 s (Marking and Meyer, 1985). It is important to determine the effective concentration of anesthetics in each fish species to provide high welfare to fish and the environment (Aydın and Barbas, 2020). Effective concentrations of anesthetics were investigated in various fish species (Cunha et al., 2010; de Oliveira et al., 2019a; Gökçek et al., 2017; Hoseini et al., 2015; Ribeiro et al., 2019; Romaneli et al., 2018; Roubach et al., 2005). However, there is only one study on *G. rufa* anesthesia in the literature, and it reported that the minimum effective concentration of clove (*Eugenia caryophyllus*) essential oil and 2-phenoxyethanol were 50 μL L^{-1} and 300 μL L^{-1}, respectively (Aydın et al., 2019). Except for clove oil and 2-phenoxyethanol, research on different anesthetic agents for *G. rufa* was not found in the literature.

According to our results, 25–50 mg L^{-1} thymol and 50 μL L^{-1} carvacrol were effective concentrations in *G. rufa* anesthesia. The effective concentrations of thymol and carvacrol in the current study were lowest than eugenol (80–160 mg L^{-1}) (Ribeiro et al., 2019), menthol (80 mg L^{-1}) (Teta and Kaiser, 2019), citronellal (800 mg L^{-1}) and linalool (1600 mg L^{-1}) (Yousefi et al., 2018b), 1,8-cineole (600–800 μL L^{-1}) (Taheri Mirghaed et al., 2018a) and myrcene (250 μL L^{-1}) (Taheri Mirghaed et al., 2018b) in other fish species. The effective concentrations in the present study are similar to other published data (Cunha et al., 2010; de Oliveira et al., 2019a, 2019b; Pereira-

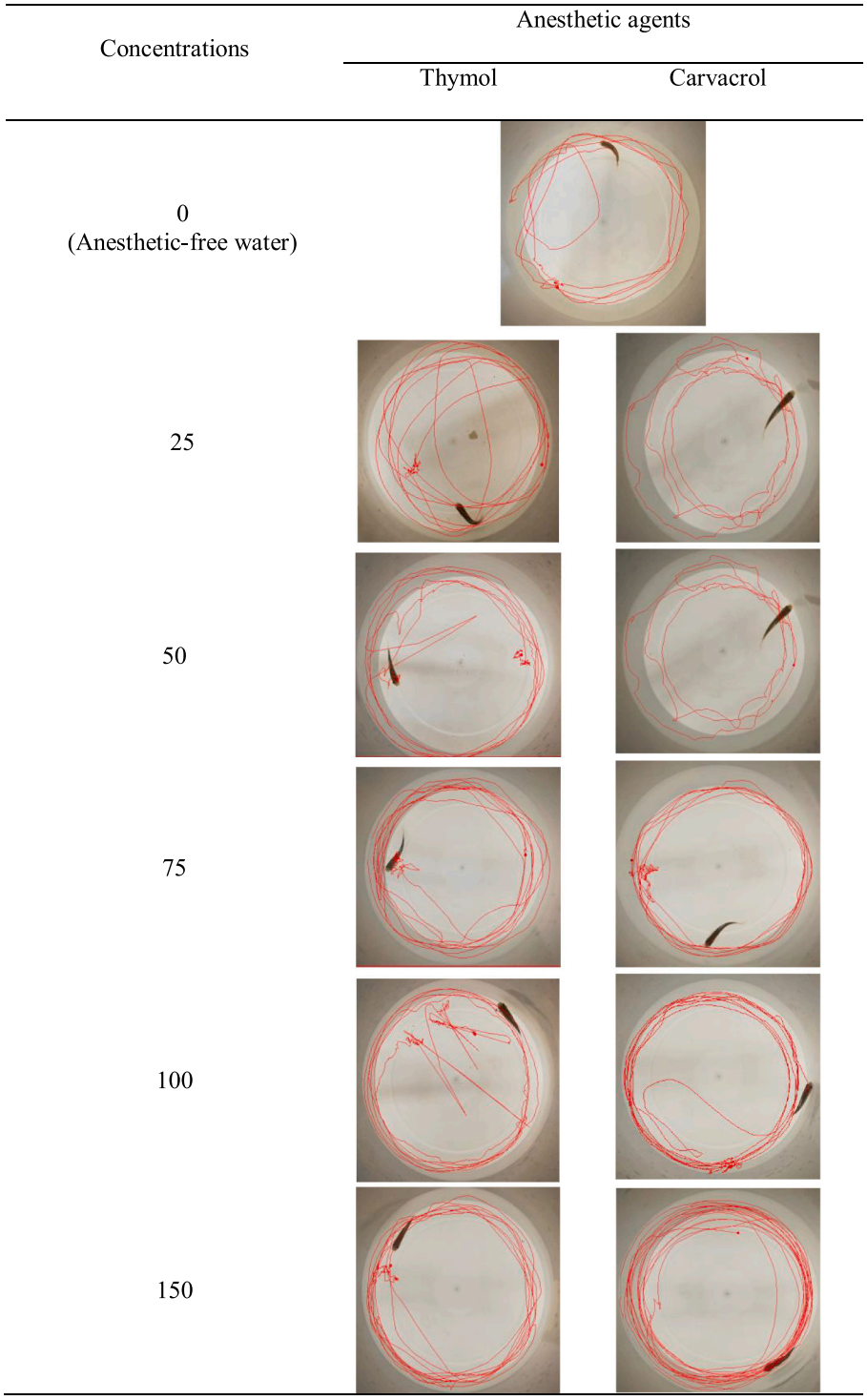

Fig. 3. Movement tracks of *G. rufa* exposed to different concentrations of thymol and carvacrol.

da-Silva et al., 2016; Roubach et al., 2005; Tarkhani et al., 2017b; Zapata-Guerra et al., 2020). Thymol anesthetized *G. rufa* within 65.6 ± 5.8 s at 100 μL L^{-1} in the current study, which may be used in aquaculture procedures, such as rapid blood collection. Taheri Mirghaed et al. (2018b) recommended of the use of 530 μL L^{-1} myrcene for rapid sampling. Yousefi et al. (2018a) found that thymol induced anesthesia in carp (*Cyprinus carpio*) within 60–850 s (recovery time 210–1200 s) at concentrations of 25–200 mg L^{-1}. Our results showed that thymol was more efficacious in *G. rufa* compared to *C. carpio*. However, it is difficult to compare anesthesia study results because of the differences in fish species, fish weight, physiological status of the fish, and water quality parameters (Aydın et al., 2019; Hoseini et al., 2015; Tarkhani et al., 2017b). The results showed a strong relationship between thymol and carvacrol concentrations and the stage 2 induction time ($R^2 = 0.9087$; $R^2 = 0.9048$, respectively), but this relationship decreased during the stage 2 recovery time ($R^2 = 0.7417$, $R^2 = 0.4896$, respectively) (Table 1). The present results are consistent with Teta and Kaiser (2019).

The use of a video tracking system to quantify and characterize the locomotor behaviors of fish under various experimental conditions, such as exposure to anesthetics, strengthen the study results (Jadot et al.,

Table 3
Electrocardiographic parameters of *G. rufa* anesthetized with different concentrations of thymol and carvacrol.

	Heart rate (beats min^{-1})	PQ interval (ms)	PR interval (ms)	QT interval (ms)
Thymol concentration ($mg\ L^{-1}$)				
50	88.2 ± 10.6^{a}	72.0 ± 10.3	90.4 ± 7.2^{b}	321.6 ± 24.6^{b}
100	73.2 ± 11.8^{ab}	68.4 ± 10.5	88.6 ± 9.5^{b}	349.2 ± 31.4^{ab}
150	57.0 ± 7.2^{b}	82.0 ± 13.6	106.6 ± 10.7^{a}	386.4 ± 43.7^{a}
Carvacrol concentration ($\mu L\ L^{-1}$)				
50	64.4 ± 7.0	69.0 ± 7.9	89.0 ± 7.4	282.6 ± 40.5
100	63.8 ± 7.5	70.2 ± 9.4	86.6 ± 9.2	291.4 ± 42.4
150	58.2 ± 6.4	71.8 ± 3.8	92.6 ± 4.6	270.1 ± 35.6

Data are expressed as the means ± SD (n=5).
Values with different superscripts in each column are significantly different ($P<0.05$).

2005). An increasing number of studies have used video tracking in aquatic species to get valuable information on toxicological agents, drugs and other study topics in recent years (Alyuruk et al., 2013; Bardera et al., 2020; Boyle et al., 2013; Hansen and Roslev, 2016; Jadot et al., 2005; Jijie et al., 2020). It is well established that anesthetic agents physiologically affect fish (Aydın and Akhan, 2020; Boaventura et al., 2020; de Freitas Souza et al., 2018; Hoseini et al., 2020; Mazandarani et al., 2017; Taheri Mirghaed et al., 2018a), but only a few studies used video tracking to assess behavioral parameters of fish during the induction of anesthesia (Nordgreen et al., 2014; Readman et al., 2013, 2017). Behavioral studies with computer-based technology can provide the most effective tools for fish welfare assessment. Therefore, we used the video tracking technology and tracking software to obtain behavioral variables of *G. rufa* related to the swimming activity, such as movement distance, swimming speed (velocity) and mobility. Table 2 and Fig. 3 show significant differences in swimming speed between thymol concentrations. Significant differences were also detected in all behavioral parameters for the carvacrol concentrations. The swimming activity of fish exposed to anesthetic baths should decrease. However, Fig. 3 shows that increasing concentrations of carvacrol increased movement distance and swimming speed. Altered behavior should be considered an indicator of stress and poor welfare in fish (Martins et al., 2012; Readman et al., 2013). Video tracking analyses showed that high concentrations of thymol ($150\ mg\ L^{-1}$) and carvacrol (100 and $150\ \mu L\ L^{-1}$) caused avoidance behaviors such as increased the distance moved. Also, as can be seen in Table 2 and Fig. 3, the fish displayed circular movement behavior with high swimming speed. According to Ashley (2007), these findings may indicate the deterioration of the fish welfare level. Readman et al. (2013) studied 2-phenoxyethanol ($0.15\ ml\ L^{-1}$), benzocaine ($50\ mg\ L^{-1}$); isoeugenol ($10\ mg\ L^{-1}$), ethyl 3-aminobenzoate methanesulfate (MS-222) ($50\ mg\ L^{-1}$) and quinaldine sulphate ($10\ mg\ L^{-1}$) as anesthetics in zebrafish (*Danio rerio*) and observed significant difference in the distance travelled and swimming speed. Readman et al. (2017) also stated that the use of MS-222 and benzocaine induced avoidance behaviors in medaka (*Oryzias latipes*). *C. carpio* showed avoidance behaviors to etomidate in the same study. This avoidance behavior in fish may be due to the irritating effects of the anesthetics on skin, gills and taste-smell receptors (Readman et al., 2017). The results of the present study determined that anesthetic concentration had a significant effect on movement distance, swimming speed, and mobility of *G. rufa*. Low concentrations of thymol ($25–75\ mg\ L^{-1}$) and carvacrol ($25–75\ \mu L\ L^{-1}$) had no adverse effect on the behavioral parameters of *G. rufa*. *O. latipes* and *D. rerio* also showed no aversion to etomidate ($1\ mg\ L^{-1}$) (Readman et al., 2017, 2013). Anesthesia with MS-222 or benzocaine had no aversive effects on carp (Readman et al., 2017). Nordgreen et al. (2014) reported that MS-222 has no effect on swimming speed in *D. rerio*. In contrast, Strungaru et al. (2019) showed that total distance and swimming speed were significantly decreased in *D. rerio* exposed to 12 and $25\ \mu g\ L^{-1}$ deltamethrin (insecticide). Readman et al. (2013) stated that the difference in behavioral reactions to the test compounds may be a function of different chemoreceptor stimulation in some cases. In addition, as shown in Table 2 and Fig. 3, it is clear that the differences in behavioral parameters are affected by anesthetic concentrations.

Electromyograms, electrocardiograms and electroencephalographs are excellent techniques to reflect the degree of environmental stress on fish physiology when fish are exposed to anesthetic agents or toxic substances (Barbas et al., 2017; Cantanhêde et al., 2020; de Souza et al., 2019; Fujimoto et al., 2018; Song et al., 2018). Barbas et al. (2017) stated there is an important knowledge gap regarding the unknown effects of most anesthetics currently used in fish on electrophysiological responses in the brain, heart and muscle. There are few studies on the electrocardiographic responses of fish submitted to anesthetic agents (Barbas et al., 2017; Cantanhêde et al., 2020; de Souza et al., 2019; Huang et al., 2010). In this study, the electrocardiographic responses of *G. rufa* was examined for the first time. Our results showed that heart rates (HRs) in the $50\ mg\ L^{-1}$, $100\ mg\ L^{-1}$ and $150\ mg\ L^{-1}$ thymol concentrations were 88.2 ± 10.6 beats min^{-1}, 73.2 ± 11.8 beats min^{-1} and 57.0 ± 7.2 beats min^{-1}, respectively. The increase in thymol concentration produced a significant decrease in HRs. However, bradycardia was not statistically significant in fish exposed to carvacrol concentrations in the current study. This difference may be related to the mechanisms of action of thymol and carvacrol on fish physiology. Similar to our findings, Barbas et al. (2017) observed that the HR in sham control was 77 ± 6 beats min^{-1}, and deep anesthesia with $600\ \mu L\ L^{-1}$ of the essential oil of citronella suppressed cardiac frequency to 50 ± 2 beats min^{-1} in tambaqui (*Colossoma macropomum*). de Souza et al. (2019) reported that HR decreased from 100 ± 9 beats min^{-1} (Control) to 57 ± 4 beats min^{-1} and 58 ± 3 beats min^{-1} during the anesthetic induction of *C. macropomum* with the essential oils of *Nepeta cataria* and propofol, respectively. Similar results were reported in *C. carpio* (Dziaman et al., 2010) and *Bryconops caudomaculatus* (Cantanhêde et al., 2020). In contrast to the results of the current study, tricaine methanesulfonate + isoflurane did not cause a reduction of HR in the *D. rerio* (Huang et al., 2010). There may be important differences in cardiac morphology between fish species, and HR is affected by the fish species and water temperature (Ma et al., 2019). Therefore, it is not easy to compare the results between different studies.

ECG analysis of the present study showed that the PR interval of fish exposed to thymol increased at the $150\ mg\ L^{-1}$ concentration. There was also a significant increase in the PR interval of *Brycon amazonicus* and *Hoplias malabaricus* exposed to inorganic mercury (Hg) (Monteiro et al., 2017). Compared to the $50\ \mu L\ L^{-1}$ thymol concentration, there was a significant QT interval prolongation in the $150\ \mu L\ L^{-1}$ concentration. Prolongation of the QT interval was also seen in *H. malabaricus* exposed to inorganic mercury (Monteiro et al., 2017). Electrocardiographic signals are also used for monitoring exposure to toxic chemicals, and the QT interval was prolonged when *D. rerio* were exposed to thallium and deltamethrin (Song et al., 2018). In contrast to the present results, Kakuta and Murachi (1997) concluded that PQ and QT intervals were shortened when *C. carpio* were exposed to 20% and 50% sewage. Ma et al. (2019) stated that there may be differences between fish species in cardiac hemodynamics due to differences in cardiac morphology, the duration of electrical impulses in the heart, and cardiac thermal sensitivity. According to Isbister and Page (2013), some drugs that are affiliated with QT prolongation in humans also led to QT prolongation in *D. rerio* (Milan et al., 2006). Knowledge of the normal and dangerous electrocardiographic parameter values of fish will help determine ideal anesthetic and effective concentration.

5. Conclusion

The results showed that 25 and $50\ mg\ L^{-1}$ thymol and $50\ \mu L\ L^{-1}$ carvacrol concentrations can be used for general aquaculture procedures. The $100\ mg\ L^{-1}$ concentration of thymol and $75\ \mu L\ L^{-1}$

concentration of carvacrol can be used for rapid sampling of *G. rufa*. Video tracking analyses showed that high concentrations of thymol (150 mg L^{-1}) and carvacrol (100–150 μL L^{-1}) caused avoidance behaviors in *G. rufa*. Anesthetic baths had a strong influence on the swimming behavior of fish. Therefore, it is important for future studies to monitor the behaviors of the fish in determining the ideal concentration of anesthetics. To ensure the welfare of fish during anesthesia, it is essential for further studies to understand the mechanisms of action of anesthetic agents on the cardiographic and behavioral parameters of fish. Determinations of the effects of anesthetics and anesthesia doses on fish heart parameters, in freely swimming fish should be the target of future studies.

Declaration of Competing Interest

The authors declared that there were no conflicts of interest.

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The Animal Experiments Local Ethics Committee from Faculty of Fisheries, Akdeniz University approved all of the experimental protocols before the study began (Decision date 04.10.2018, decision no 002 and decision date 12.03.2019, decision no 012).

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