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Effects of Temperature, Fish Size and Dosage of Clove Oil on Anaesthesia in Turbot (*Psetta maxima* Linnaeus, 1758)

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Abstract

The effects of four concentrations of clove oil (150, 300, 450 and 600 mg L^{-1}) as ananaesthetic substance at three temperature levels (10, 15 and 20°C) and three fish size (as weight) groups (57.0±11.3, 103.0±11.9 and 457.0±89.8 g) on induction and recovery times in turbot (*Psetta maxima*) were investigated in this study. The fish were individually exposed to each clove oil bath in 10, 20 or 50L buckets with respect to fish size and were recovered in a 500-L tank with running seawater. Temperature, fish size and clove oil concentrations were found to have significant effects on induction and recovery times in turbot ($P<0.01$). Induction and recovery times were both less than half when the temperature was increased from 10 to 20°C ($P<0.01$). The duration to reach surgical anaesthesia and recovery times of small size turbot varied in relation to temperature, but were generally shorter than big size turbot ($P<0.01$). Overall, the increase of clove oil concentration from 150 to 600 mg L^{-1}decreased the induction time by a factor of 2.3 but on the contrary, prolonged the full recovery time by a factor of 1.92. The surgical anesthesia was attained in all the groups under 4.45 min at 150 mg L^{-1}or even under 2 min at 600 mg L^{-1} clove oil concentration. However, the time of recovery ranged from 4.32 min (150 mg L^{-1}) to 8.29 min (600 mg L^{-1}). Based on our results, the clove oil concentrations around 180-220 mg L^{-1} appeared to be adequate to be used for fast anaesthesia and relatively short recovery time for turbot. Mean effective concentration (EC50) of clove oil was calculated as 190.00 ± 10.34 mg L^{-1} with 95% confidence limits for overall three temperature and fish sizes. This study has demonstrated that clove oil can be safely and effectively used in the anaesthesia of turbot.

Keywords: *Psetta maxima,* clove oil, temperature, effective concentration, fish size.

Karadeniz Kalkan Balığının (*Psetta maxima* Linnaeus, 1758) Karanfil Yağı ile Bayıltılmasında Sıcaklık, Balık Büyüklüğü ve Konsantrasyonun Etkileri

Özet

Bu çalışmada, Karadeniz kalkan balığının karanfil yağı ile bayıltılmasında sıcaklık (10, 15 ve 20°C), balık büyüklüğü (57,0±11,3, 103,0±11.9 ve 457,0±89,8 g) ve konsantrasyonun (150, 300, 450 ve 600 mg L^{-1}) bayılma ve ayılma sürelerine etkileri araştırılmıştır. Çalışmada balıklar tek tek 10, 20 ve 50 L'lik kaplarda bayıltılmış ve sürekli su değişimi sağlanan 500 L tankta ayıltılmışlardır. Su sıcaklığı, balık büyüklüğü ve karanfil yağı konsantrasyonunun bayılma ve ayılma sürelerini etkilediği belirlenmiştir (P<0,01). Su sıcaklığı 10°C'den 20°C'ye yükseldiğinde bayılma ve ayılma süreleri azalmıştır (P<0.01). Küçük balıkların bayılma ve ayılma süreleri su sıcaklığına bağlı olarak değişmesine rağmen, genel olarak büyük balıklardan daha kısa olmuşlardır. Karanfil yağı konsantrasyonu 150 mg L^{-1} den 600 mg L^{-1}'ye çıkarıldığında bayılma süresi 2,3 kat azalmış, buna karşılık ayılma süresi 1,92 kat artmıştır. Bütün gruplarda 150 mg L^{-1} konsantrasyonda 4,45 dakika ve 600 mg L^{-1} konsantrasyonda 2 dakikanın altında cerrahi anesteziye ulaşılmıştır. Ayılma süresi 4,32 dakika (150 mg L^{-1}) ile 8,92 dakika (600 mg L^{-1}) arasında değişmiştir. Elde edilen sonuçlara göre, Karadeniz kalkan balığının hızlı bayılması ve nispeten kısa sürede ayılması için 180-220 mg L^{-1} karanfil yağı konsantrasyonlarının uygun seviyeler olduğu görülmektedir. Karanfil yağının ortalama etkili konsantrasyonu (EK50) %95 güven aralığında 190,00 ± 10,34 mg L^{-1} hesaplanmıştır. Bu çalışma, Karadeniz kalkan balığının bayıltılmasında karanfil yağının güvenle kullanılabileceğini göstermiştir.

Anahtar Kelimeler: *Psetta maxima,* karanfil yağı, sıcaklık, etkili konsantrasyon, balık büyüklüğü.

Introduction

Anaesthesia has been used for long time in the world's aquaculture activities to minimise stress on fish and thus reducing suffering and injuries during transportation, breeding, capturing, handling and conducting painful procedures such as tagging, vaccination and injection (Marking and Meyer, 1985; Rossand Ross, 2008). These substances help to induce a calming effect followed by a sucessive loss of equilibrium, mobility, consciousness as well as reflex action in fish.

The most commonly used fish anaesthetics are MS-222 (tricaine methanesulphonate), benzocaine, carbon dioxide, clove oil, AQUI-S, quinaldine, quinaldine sulphate, 2-phenoxyethanol, metomidate and etomidate (Marking and Meyer, 1985; Ross and Ross, 2008). The ideal anaesthetic agent should produce rapid anaesthesia (1-5 min) and permit a quick recovery (<5 min) as well as it should be easy to handle, non-toxic to fish, human and environment, resulting in low tissue residues, easily soluble in water and commercially available at affordable prices (Marking and Meyer, 1985; Ross and Ross, 2008). Use of clove oil as an anaesthetic caninducea shorter induction but longer recovery than the recommendations above (Sladky *et al*., 2001; Detar and Mattingly, 2004). Advantages of clove oil are its efficiency at a range of temperatures, easy availability, low cost as well as safety for both fish and handlers (Detar and Mattingly, 2004).

In recent years, the use of clove oil being a relatively new as a fish anaesthetic has gained popularity among others. It is extracted from the flowers, leaves and stalks of the clove tree (*Eugenia aromaticum* or *Eugenia caryophyllata*) (Soto and Burhanuddin, 1995; Ross and Ross, 2008). Its main active ingredients include eugenol (76.8-88.58%), eugenyl acetate (1.2-5.62%) and β-caryophyllene (1.39-17.4%) (Jirovetz *et al*., 2006; Chaieb *et al*., 2007). Eugenol has a multitude of properties making it useful in a wide variety of applications, including as an antioxidant (Kramer, 1985; Pulla and Lokesh, 1992), antifungal (Kamble and Patil, 2008; Hoskonen *et al.* 2015), antibacterial (Karapmar and Aktug,1987; Kouidhi *et al*., 2010) or as an antiparasitic agent (Machado *et al*., 2011). Several studies have shown that clove oil is an effective agent in the sedation of larvae (Akbulut *et al*., 2011a), fry (Endo *et al*., 1972; Woolsey *et al*., 2004; Akbulut *et al*., 2012a), juvenile (Keene *et al*., 1998; Uçar and Atamanalp, 2010; Akbulut *et al*., 2011b) and adult fish of various species (Hikasa *et al*., 1986; Wagner *et al*., 2002; Hoskonen and Pirhonen, 2004). Among flatfish species, clove oil efficacy has been tested on the sole *Solea senegalensis* (Weber *et al*., 2009) and on the flounder *Platichthys flesus* (Akbulut *et al*., 2012b), but it has never been tested on the anaesthesia of turbot *Psetta maxima*with relation to temperature and fish size. Therefore, the effects of temperature, fish size and concentration levels of clove oil on induction and recovery times were for the first time investigated and its effective concentrations were calculated for this species in this study.

Materials and Methods

Turbot raised from eggs in the Marine Fish Hatchery of Central Fisheries Research Institute, Trabzon (Turkey) were used in the study. Three fish size-groups weighted as [57.0±11.3 g (15.9±1.57 cm total length, TL), 103.0±11.9 g (19.4±0.90 cm TL), and 457.0±89.8 g (31.1±1.95 cm TL)] were separated into 9 fiberglass tanks (0.4 m^3) and each size group was reared at constant 17 ‰ salinity in three different experimental temperatures (10, 15 and 20 ˚C) for three weeks prior to the tests. A pelleted diet (containing 44% crude protein; 18% raw fat; 10.0% ash; 3.5% cellulose; and 17.2 $MJkg^{-1}$) at a rate of about 1% of body weight per day with two meals was given to the fish until the onset of the tests. The fish were starved for 24 h prior to the tests.

In addition, the size and temperatures, four concentrations of clove oil (150, 300, 450 and 600 mg L^{-1}) were tested to determine combined effects of these factors on induction and recovery times of anaesthesia in turbot. A total of 360 fish were used in the whole study and 10 fish in each of the treatment combination. During the trials, the fish from each of the three fish size-groups were individually exposed to each of the treatment combination. The water temperature in the buckets (10, 20 or 50 L) was held constant during the experiments by placing them in thermostatically controlled water baths. The measurement of induction and recovery times was modified from earlier studies (Schoettger and Julin, 1967; Summerfelt and Smith, 1990; Keene *et al*., 1998) and described in Table 1. Induction and recovery times of each fish were measured to the nearest second.

Commercially available clove oil in 20 mL glass vials at concentration of 0.92 kg L^{-1} (Kardelen Tarım Ürünleri Ltd., Ankara, Turkey) was used in this study. The stock solution was prepared by dissolving clove oil in 95% ethanol (1:10 ratio) as described by Anderson *et al.* (1997) to facilitate mixing. Ethanol has no known anaesthetic effects on fish at such low concentration (Anderson *et al*., 1997; Cho and Heath, 2000). The clove oil stock solution was added to test water 5–6 min prior to the introduction of the fish, in order to allow to complete dissolution and mixing of the anaesthetic.

When the fish reached the surgical anaesthetic stage (S), they were immediately netted out from the anaesthetic bath (Table 1). After measurement of body weight (to 0.01 g) and length (to 0.1 cm), the fish were immediately placed into

Table 1. The stages of anaesthesia used in the study

Phase of Anaesthesia	Stage	Description	Notable behaviour of fish
Induction	I-1	Sedation	Fish swimming, reaction to external stimuli.
	I-2	Loss of equilibrium	Swimming ability stops, partial loss of equilibrium, reaction to external stimuli.
Surgical Anaesthesia	S	Unconscious	No movement, completeloss of equilibrium and reflex activity, no reaction to external stimuli, slow and irregular opercular ventilation.
Recovery	R-1	Motion	Motion perception and presence of reaction to external stimuli
	R-2	Regain of equilibrium	Partial recovery of equilibrium, regular opercular ventilation, reaction to strong external stimuli, swimming disorder.
Normal			Recovery of equilibrium, fish swimming.

a flow through recovery tank filled with 400 L seawater. Once recovered, the fish were grouped in 300 L tanks and monitored for survival and behaviour for 48 hours. During the trials, dissolved oxygen level never fell below 5.5 mg L^{-1}. Total loss of equilibrium in turbot was assumed as the inability of the fish to return to its normal position when turned upside down, while in the case of partial loss of equilibrium, the fish retained some movements (see Table 1; Weber *et al*., 2009).

Statistical Analysis

After checking the data for normality by Shapiro–Wilk's test and homogeneity of variances by Levene's test, an analysis of variance (two-way ANOVA) was performed to compare the groups in SPSS software version 16.0 for windows. Any significant difference was further tested by Tukey's multiple comparison test (Zar, 1984). The effective concentration (EC_{50}) values for optimal anaesthesia were determined by the probit analysis of Pearson Goodness of Fit Test (Finney, 1971). A multiple regression analysis of EC_{50} with respect to temperature and fish size was calculated and tested by using the coefficients obtained from regression analysis (P<0.05).

Results

In general, regardless of the fish size and concentrations of clove oil tested in this study, the induction and recovery times were both less than half when the temperature was increased from 10 to 20°C (P<0.01, Table 3 and Table 4). The duration to reach surgical anaesthesia and recovery time from the anaesthesia of small size turbot exposed to clove oil varied in relation to temperature, but were generally shorter than big size turbot (P<0.01, Table 3). Overall, the increase of clove oil concentration from 150 to 600 mg L^{-1} significantly decreased the induction time by a factor of 2.3, but on the contrary prolonged the full recovery time by a factor of 1.92 (Table 3 and Table 4).

When the clove oil was tested at four concentrations (150, 300, 450 and 600 mg L^{-1}) in turbot of average weights of 57 - 457 g at three water temperatures (10, 15, 20°C), full anesthesia was attained in all the groups under 4.45 min (150 mg L^{-1}) or even under 2 min (600 mg L^{-1}). However, the time of recovery ranged from 4.32 min (150 mg L^{-1}) to 8.29 min (600 mg L^{-1}) (Table 3 and Table 4). Based on our results, the clove oil concentration between180-220 mg L^{-1} appeared to be adequate to be used for fast anaesthesia and relatively short recovery time for turbot. Joint effects of temperature x fish size, temperature x concentration, fish size x concentration, and temperature x fish size x concentration on induction and recovery times were all found to be statistically significant (P<0.05, Table 3 and Table 4). Hence, when mean effective concentration (EC_{50}) of clove oil was calculated, the EC_{50} value of 190.00±10.34 mg L^{-1} with 95% confidence limits was found for turbot for overall three temperature and fish

Table 3. Interaction of temperature, fish size and concentration on induction times (in seconds, s) of turbot exposed to clove oil (mean ± sd)

	Induction time (s)				
Temperature (°C)	10	15	20		P
	224.0±10.58^a	176.0±6.59^b	100.3±4.06^c		0.01
Fish size (g)	57	103	457		
	156.1±9.35^a	168.0±8.65^a	183.8±9.33^b		0.01
Dose (mg L^{-1})	150	300	450	600	
	267.0±13.68^c	166.3±5.95^b	130.0±5.81^a	116.1±6.19^a	0.01
Interaction			F	DF	p
Temperature (˚C) x Fish size (g)			11.289	4	0.00
Temperature (˚C) x Dose (mg L^{-1})			19.359	6	0.00
Fish size (g) x Dose (mg L^{-1})			3.039	6	0.01
Temperature (˚C) x Fish Size (g) x Dose (mg L^{-1})			1.355	12	0.19

Means sharing the same superscript letters in same row are not significantly different from each other.

Table 4. Interaction of temperature, fish size and concentration of clove oil on recovery times (in seconds, s) of turbot exposed to clove oil (mean ± sd)

	Recovery Time (s)				
Temperature ˚C)	10	15	20		p
	481.0±17.92^c	355.1±15.83^b	206.0±6.82^a		0.01
Fish size (g)	57	103	457		
	334.2±16.95^a	336.0±16.75^a	388.1±19.89^b		0.01
Dose (mg/L)	150	300	450	600	
	259.5±12.20^a	282.3±15.26^a	371.2±17.06^b	497.2±25.89^c	0.01
Interaction			F	DF	p
Temperature (˚C) x Fish size (g)			5.266	4	0.00
Temperature (˚C) x Dose (mgL^{-1})			13.457	6	0.00
Fish size (g) x Dose (mgL^{-1})			4.458	6	0.00
Temperature (˚C) x Fish Size (g) x Dose (mgL^{-1})			2.310	12	0.01

Means sharing the same superscript letters in same row are not significantly different from each other.

sizes. The combined effect of temperature and fish size had significant effects on EC_{50} as described by the following equation:

$$EC_{50} = 582.3 - 23.1T + 0.2S$$

Where EC_{50}: Effective concentration (mg L^{-1}), T: temperature (°C) and S: fish size (g). The coefficients in the above equation were obtained through analysis of regression ($r^2 = 0.79$, $F_{2,6} = 11.05$, $P<0.05$). One fish of 57 g size-group and two fish of 103 g size-group died at the 20°C in 600 mg L^{-1} trials.

Discussion

The anaesthetic effects of clove oil have been studied in many fish species (Endo *et al*., 1972; Hikase *et al*., 1986; Weber *et al*., 1999; Prince and Powell, 2000; Hoskonen and Pirhonen, 2004; Akbulut *et al*., 2011 a, 2012b) but the current research has for the first time evaluated the use of clove oil at combinations at different temperatures, fish sizes and the clove oil concentrations to immobilise the turbot. The effective concentration (EC_{50}) of clove oil for turbot was calculated as 190±10.34 mgL^{-1} in the range of rearing temperatures and fish sizes tested in our study. The EC value for turbot is found to be higher than for some previously documented findings for other fish species *e.g.* 30 mg L^{-1} for the rainbow trout *Oncorhynchus mykiss* (Prince and Powell, 2000), 50 mg L^{-1} for the sockeye salmon *O. nerka* (Woody *et al*. 2002), 50 mg L^{-1} for the gold fish *C. auratus* (Perdikaris *et al*., 2010) and for the African catfish *Clarias gariepinus*, (Öğretmen and Gökçek, 2013), whilst lower than for some other species *e.g.* the flounder *P. flesus* (Akbulut *et al*., 2012b), the Russian sturgeon *A. gueldenstaedtii* (Akbulut *et al*., 2011b), the Persian sturgeon *A. persicus* (Imanpoor *et al*., 2010) and the Siberian sturgeon *A. baerii* (Akbulut *et al*., 2012a), which ranged between 400 and 750 mg L^{-1}.

In general, highwater temperatures enhance anaesthetic efficacy of clove oil by shortening induction time in the steelhead fry *O. mykiss* (Woolsey *et al*., 2004), the gilthead sea bream *Sparus aurata* (Mylonas *et al*., 2005), the common carp *C. carpio* (Hikasa *et al*., 1986) and the flounder, *P. flesus* (Akbulut *et al*.,2012b). Hamackova *et al.* (2006), studied with perch (*Perca fluviatilis*), reported full anesthesia after 7.57 min at 12.5°C while 6.98 min at 17.5°C, and/or 6.05 min at 12.5°C and 3.73 min at 20.0°C after the exposure to clove oil. The time of recovery ranged from 6.06 min at 12.5°C to 9.21 min at 15.0°C, and from 3.69 min at 20.0°C to 7.44 min at 12.5°C. Hamackova *et al*., (2006) also report that induction timeof fish exposed to clove oil is strongly influenced by water temperature. Optimal concentrations for clove oil to anaesthetize the marine medaka (*Oryzias dancena*) were reported as 125 mg L^{-1} at 23 °C, 100 mgL^{-1} at 26 °C and 75 mgL^{-1} at 29 °C. Similar to the above findings, an increase of temperature from 10 to 20ºC has also more than halved induction (from 3.73 to 1.67 min) and recovery times (from 8.02 to 3.43 min) in turbot fry in our study. Faster induction and recovery times displayed by fish at higher temperatures have been attributed to positive relationship between temperature and metabolism (Mylonas *et al.*, 2005).

Perdikaris *et al*. (2010) observed a size-relative difference in induction time of goldfish, and stated that rainbow trout and goldfish could recoverwithin 6 min after anaesthesia at 150 mgL^{-1} clove oil. Park *et al*. (2011) reported that induction and recovery times in medaka were both significantly shorter for smaller fish than for larger fish. Our results on induction and recovery times corroborate withthe above studies in that big size-turbot (457 g) had also significantly longer induction (3 min) and recovery times (about 6.5 min) compared to smaller fish (57-103 g) ($P<0.01$), which exhibited an average induction time of 2.6-2.8 min and recovery time of 5.6 min. The main reason for shorter induction and recovery times measured at smaller size fish could be due to faster ventilation rate and hence metabolism in these animals (Mylonas *et al*., 2005). However, the size-effects on induction and recovery times appear to species-specific (Hoskonen and Pirhonen, 2004).

An increase of clove oil concentration from 150 to 600 mg L^{-1} shortened the induction time but, on the contrary, prolonged the recovery time by more than about two-folds in turbot. The effects of high concentrations of clove oil were more evident on the smaller fish size and at the low temperature levels. The fish exposed to four concentrations of clove oil (150, 300, 450 and 600 mg L^{-1}) of average weight 57 - 457 g at three water temperatures (10, 15, 20°C) attained full anesthesia in all the groups in less than 4.45 min (150 mg L^{-1}) or even under 2 min (600 mg L^{-1}). However, the time of recovery inversely ranged from 4.32 min (150 mg L^{-1}) to 8.29 min (600 mg L^{-1}). All the clove oil concentrations tested in our study (150-600 mg L^{-1}) induced total loss of equilibrium in turbot but induction and recovery times were both significantly affected by the dosage. In a nutritional study, Bonaldo *et al.* (2014) used 70 mg L^{-1} clove oil concentration (recommended for *S. senegalensis* by Weber *et al*., 2009) to sedate turbot juveniles (20-69 g) and the fish reachedfull anaesthesia in 3 min at 18°C water temperature. In our study, at an average fish size of 57 g and at 20°C water temperature, we obtained full anaesthesia under 2.35 min. In general, based on our data, the adequate concentration providing shortest induction and recovery times forthe full surgical anaesthesia of turbot appeared to be between180-220 mg L^{-1}.

Despite some disadvantages that have been reported about the use of clove oil as a fish anaesthetic by a few authors (Hoskonen and Pirhonen, 2004), most have well acknowledged that it is safe and an effective fish anaesthetic. In the United States clove oil is not permitted by the FDA for food fishes, even though it is "generally regarded as safe", In contrast to some other anaesthetics, a withdrawal period for clove oil is considered unnecessary, as it does not pose any environmental hazard (Cho and Heath, 2000). There are several advantages of using clove oil as ananaesthetic agent in fish.It is a natural product and is considered neither toxic nor carcinogenic to humans and other animals (Fisher *et al*., 1990; Zheng *et al*., 1992; Lee and Shibamoto, 2001). Furthermore, its low-cost and easy availability make this substance as a good substitute for chemical anaesthetics. As we obtained acceptable induction and recovery times and negligible mortality even at the very highest tested concentration (600 mg L^{-1}) in our study, we have also concluded that clove oil can be effectively used as ananaesthetic especially at the levels lower than 200 $mgL^{-1}$for the turbot of various sizes.

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