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Sedative Effect of Clove Oil and 2-Phenoxyethanol on Marine Clownfish (*Amphiprion ocellaris*) and Freshwater Swordfish (*Xiphophorus helleri*)

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Authors' Contribution
MAH organized and conducted research, wrote the text and made statistics. CS helped in the research. SS helped in the statistics. KF helped during the trial and writing.

Key words
Clownfish, Amphiprion ocellaris, Swordfish (Xiphophorus helleri), Anaesthesia, Clove oil, 2-phenoxyethanol,

ABSTRACT

This study's objective was to determine effect of two different anaesthetics 2-phenoxyethanol and clove oil on the sedation of some aquarium fish species. The induction and recovery times were determined at five stages of melanistic and not melanistic clownfish (*Amphiprion ocellaris*) and different sexes of Swordfish *(Xiphophorus helleri*). Clownfish were tested with 0.4, 0.6 and 0.8 ml/l phenoxyethanol and 0.5, 1 and 1.5 ml/l clove oil. Swordfish were tested with 1, 3 and 5 ml/l phenoxyethanol and 2, 4, 6 ml/l clove oil. For short-term application the safe dose for clownfish was 0.4 ml/l phenoxyethanol and 1 ml/l clove oil. For swordtail fish, the ideal amount of phenoxyethanol was 5 ml/l and 6 ml/l clove oil. These safe doses reduce handling stress and mortality.

INTRODUCTION

Anesthetic substances, which are used in every stage of aquaculture, decrease the level of stress on fish during applications. This situation removes negative effects, physical traumas and losses (Summerfelt and Smith, 1990; Soto and Burhan-ud-Din, 1995; Munday and Wilson, 1997; Ross and Ross, 2008). An ideal anesthetic should induced in 3 min and recover in 5 min or less for aquaculture studies. It should not endanger animal and human health. People usually prefer low costs anaesthetic agent (Marking and Meyer, 1985; Ross and Ross, 2008).

Clove oil and 2-phenoxyethanol are among the mostly preferred local anesthetics in some aquaculture practices. 2-Phenoxyethanol is a colorless, slightly aromatic and oily liquid which can be applied directly and used for fish extensively (Ross and Ross, 2008; Tamaru *et al.*, 2001). Clove oil is extracted from the clove plant. It is aromatic, slightly oily, local anaesthetic. This agent can dissolve in temperate-water easily, but it should be used with ethanol for a cold-water condition (Park *et al.*, 2008). Clove oil has many benefits such as being antibacterial, antiviral, and natural analgesic agent. Moreover, it is economic and is not being harmful to animal health. That's why make clove oil a widely preferred substance (Keene *et al.*, 1998; Tort *et al.*, 2002; Ross and Ross, 2008).

Clownfish species are great importance among marine aquarium fish. Within recent years, the financial value of ornamental fish has increased due to the increasing demand for sea aquariums (Olivotto *et al.*, 2003; Avella *et al.*, 2007; Ajith Kumar *et al.*, 2010; Dhaneesh *et al.*, 2012). They were collected from their coral reefs until quite recently, but they have been started to be produced in closed systems today which include all the conditions for fish growth phases (Allen, 1980; Haschick, 1998; Pomeroy and Balboa, 2004; Gopakumar *et al.*, 2001). The cultured clownfish reaches to 5 cm and costs $ 0.53 and is sold at a price of $ 2.25 within 120 days (Pomeroy and Balboa, 2004). This fish was the most common ornamental species covering 15.6% of the total number of fish exported worldwide during 1997–2002 (Wabnitz *et al.*, 2003). Cultivation of tropical marine aquarium fish, which are a significant part of aquaculture studies, is an important agriculture and industry brunch. It is known that ornamental marine aquarium fish are exposed to manipulation in every phase of cultivation especially before their international transportation. There are many research about this fish such as broodstock management and spawning (Kumar and Balasubramanian, 2009), biology (Yasir and Qin, 2009), feeding (Yasir and Qin, 2010; Olivotto *et al.*, 2011).

Studies on sedation and anesthesia are not abundant in the clownfish *Amphiprion* sp. Therefore, in this study the efficacy of two different sedative and anaesthetic substances on clownfish which show melanistic and not melanistic growth has been identified. It is expected to

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provide helpful information on the sedation procedure of Melanosis and not Melanosis in clownfish in the aquaculture facilities.

Swordtails have often been used in behavior ecology, genetics, biology (Kayım et al., 1999) and biogeography (Gutierrez-Rodriguez et al., 2007) and is considered an ideal species for the toxicological studies of endocrine-disrupting chemicals (Kwak *et al*., 2001) due to its great advantages, which include the small size of species and the ease of raising them, a short generation time and distinct sexual characteristics . Besides all these, swordtail fish, which are of great importance among aquarium fish, are also among species with financial value due to their colors and tail structures (Tamaru *et al*., 2001). In this study, effort was made to explain in a detailed manner the reaction of male and female swordtail fish to anesthetic applications carried out with 2-Phenoxiethanol and clove oil, which enable many scientific studies to be carried out.

MATERIALS AND METHODS

Anaesthetic agents

In the tests, commercial phenoxyethanol and clove oil were used. Phenoxyethanol was purchased from Sigma-Aldrich. Purity of the phenoxyethanol was reported as 99.9%. Clove oil was purchased from a herbalist (Kardelen Comp., Turkey). The content of the clove oil was analyzed in the laboratory of Pharmacy, Faculty of Ege University in Turkey. Its content was reported as 96.1% eugenol, 2.41% Caryophyllene, 0.29%, a-Selinene, 0.27 Isochiapin B.

Experiment fish and keeping conditions

After 210 clownfish and 420 swordfish were brought from a commercial ornamental fish breeders facility (Orta Dogu Aquarium Comp., Izmir); all of them were randomly placed in five glass aquariums (47x37x29 cm) until the experiment day. All aquariums water temperature were kept 23±0.5°C, pH were 8-8.5 for clownfish, 7-7.5 for swordfish. Illumination was used in the aquariums for 8 h. The air was delivered to each aquarium through central air engine. Fish were fed with commercial fish food ad libitum every day. Feces were removed immediately.

The clownfish were separated according to their body color; melanosis (blackish brown color) and not melanosis (light reddish color). The mean weight and total length were measured 3.21± 0.62 g and 5.36±0.38 cm of melanosis group, respectively. The not melanosis group´s measurements were 3.47± 0.65 g of mean weight and 4.63±0.60 cm of mean total length. Swordfish were measured 3.15±0.85 g mean weight and 6.72 ±0.24 cm total length for male; 3.67±0.86 g mean weight and 4.36±0.58 cm total length for female, respectively.

Experiment design

Feeding was stopped before 24 h for experiment day. The sedative agent phenoxyethanol was mixed with one-litre water for induction in a small observation aquarium. Clove oil was used after being dissolved in ethanol at a rate of 1/9 (1:10 ratio of clove oil: ethanol) as described by Anderson *et al*. (1997). Melanosis and not melanosis groups were exposed to 3 doses of clove oil (0.5, 1 and 1.5 ml/l) and 3 doses of phenoxyethanol 0.4, 0.6 and 0.8 ml/l. Swordfish were exposed to 1, 3, and 5 ml/l doses of phenoxiethanol and 2, 4 and 6 ml/l clove oil.

Ten fish were used for every agent dose and replicates. Induction and recovery behaviour were recorded with a

Table I.- Description of the respective stage of anaesthesia and recovery in clownfish (modified from Ross and Ross, 2008).

	Stage	Description	Physiological and behavioural signs
Induction	A	Light sedation	Slow swimming, decreased reactivity to external stimuli, body balance is normal.
	B	Deep sedation	Suppression of a complete loss of reactivity except strong external stimuli operculum slight decrease in the movement partial body balance loss.
	C	Light anaesthesia	Low turning to one side but still reaction to external stimuli particularly in the fish, opercular movements continued.
	D	Deep anaesthesia	Lying on one side without movement, opercular movements are very slow usually difficult to see.
	E	Surgical anaesthesia	Total loss of reaction to even massive stimulation.
Recovery	A	Deep anaesthesia	Body immobilized but opercular movements just starting.
	B	Anaesthesia	Regular opercular movements.
	C	Deep sedation	Gross body movements beginning.
	D	Light sedation	Preanesthetic appearance.
	E	Normal	Equilibrium regained.

video camera. The criteria of induction and recovery were shown in Table I. The experiment was repeated three times for each dose. Measurements were made by using a stop-watch and every phase transition was identified separately for induction and recovery. After the experiment, the fish were placed in aquaria filled with clean water and aerated with an air pump. The observation were continued until they intake food. The experimental fish were monitored for any abnormal behaviour and or mortality during a week.

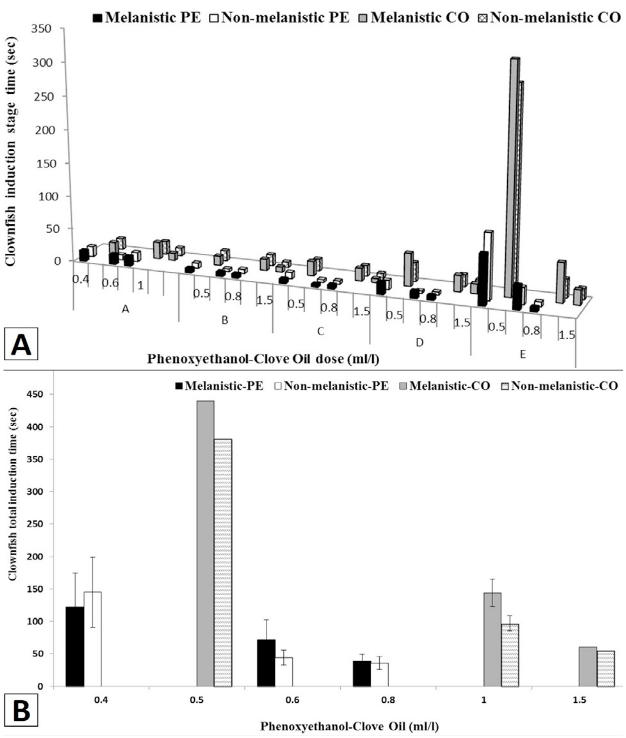

Fig. 1. Induction stage time (A) and total induction time (B) of melanistic and not melanistic clown fish which were applied phenoxyethanol and clove oil (Mean±SD).

Statistical analysis

All the statistical analysis was performed using SPSS 15.0 for Windows. Differences between agent doses consisted with Kruskal-Wallis. Statistical comparisons were made between the clove oil and phenoxyethanol exposed groups within melanosis and not melanosis groups and then male-female swordfish groups by using the non-parametric Mann-Whitney U-test.

RESULTS

It was found out that there were no deaths in the week following the test of the two anesthetic substances and it was found that the fish fed in the same day. During the procession, the fish reacted to anesthetics differently in terms of swimming. In this context, the fish put in the phenoxyethanol container swam rapidly due to abnormal swimming behavior. In the group to which was applied clove oil, the fish did not show abnormal behaviors and induced following the normal swimming behavior when put in water.

Clownfish

The induction stage time (Fig. 1A) and total induction time (Fig. 1B) of clownfish in phenoxyethanol and clove oil application is shown in Figure 1; whereas, Figure 2 shows recovery stage times (Fig. 2A) and total recovery times (Fig. 2B) of clownfish in phenoxyethanol and clove oil application.

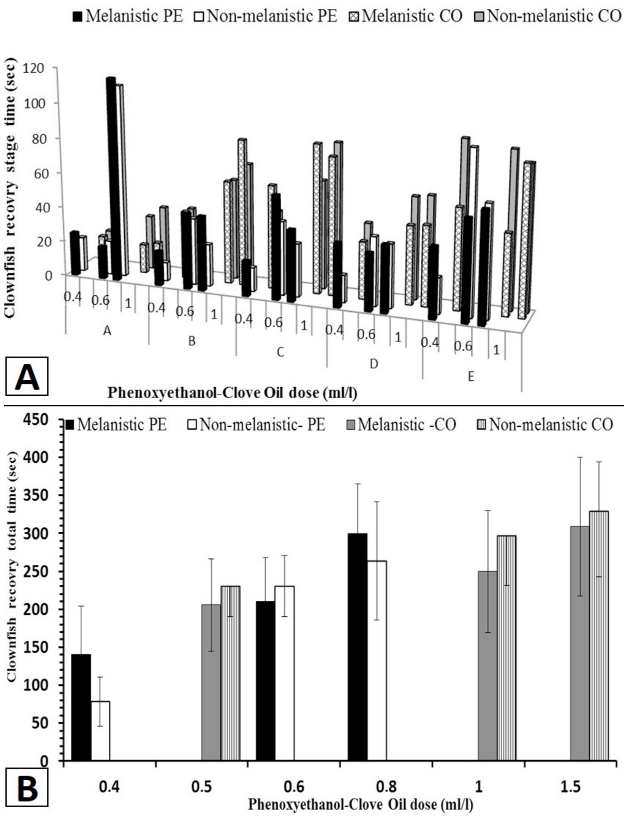

Fig. 2. Recovery stage times (A) and total recovery times (B) of not melanistic and melanistic clownfish which were applied phenoxyethanol and clove oil (Mean±SD).

In the assessment, it was found out that there were differences between doses (0.4, 0.6, 0.8 ml/L) regarding the total induction time of both melanistic and not melanistic groups ($p<0.05$). Also when melanistic and not melanistic clownfish were compared by each test dose, a difference was determined between the total induction times in 0.4 and 0.6 ml/L. test dose ($p<0.05$), but there was no difference between the dose groups in 0.8 ml/L test dose ($p>0.05$).

When the recovery times of the fish were assessed, it was also found out that there was a difference in total recovery times of both not melanistic clownfish and melanistic clownfish ($p<0.05$). Together with this, when melanistic and not melanistic clownfish were compared by each test dose, a difference was found in 0.4 ml/L test dose ($p<0.05$), whereas there was no difference in 0.6 and 0.8 ml/L test dose ($p>0.05$).

In the application of clove oil, the times of induction phase transition of melanistic and not melanistic fish were shown in Figure 1A and total induction times were shown in Figure 1B. Similarly, times of recovery phase transition were shown in Figure 2A and total recovery times were shown in Figure 2B.

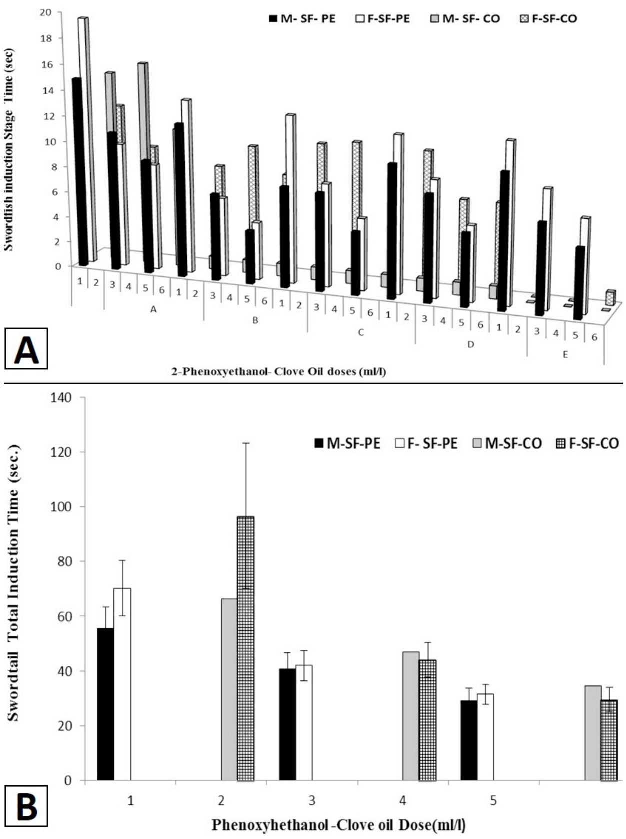

Fig. 3. Intermediate induction stage time (A) and total induction times (B) of female and male swordtail fish which were applied to 2-phenoxiethanol and clove oil (Mean±SD).

In the clove oil test, there was a difference between dose (0.5, 1, 1.5) in terms of total induction times of both melanistic and not melanistic clownfish ($p<0.05$). Also when melanistic and not melanistic clownfish were compared by each test dose, there was not a difference in 0.5 ml/L dose in terms of total induction times ($p>0.05$), but in 1 and 1.5 ml/L test dose a difference between the groups was identified ($p<0.05$).

When the recovery times of the fish were assessed, a difference between test dose in terms of total recovery times of both melanistic and melanistic clownfish was found ($p<0.05$). Also, when recovery of melanistic and melanistic clownfish was compared, it was found that the difference in the group which was applied 0.5 ml/L. The dose was insignificant; however there was a difference between recovery times the individuals in the group which was applied to 1 and 1.5 ml/L. ($p<0.05$).

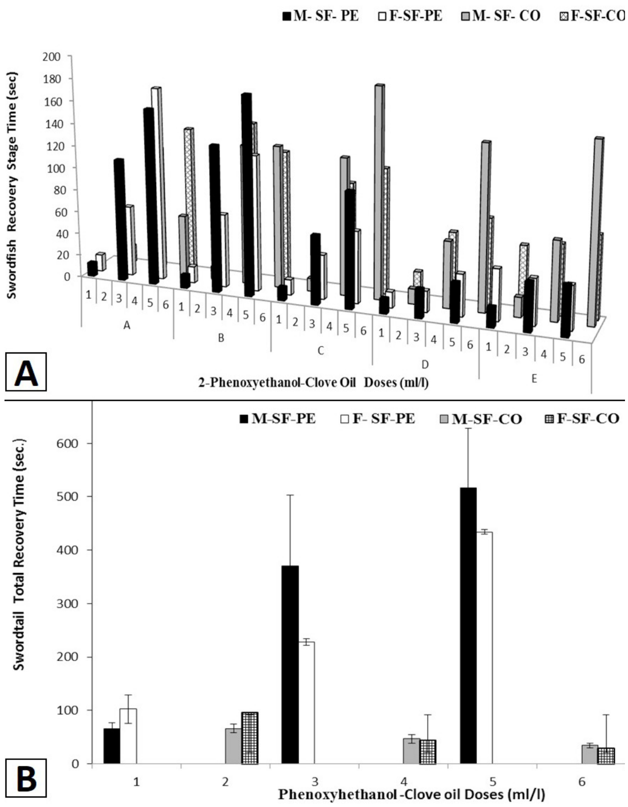

Fig. 4. Intermediate recovery stage times (A) and total recovery times (B) of female and male swordtail fish which were applied to phenoxyethanol and clove oil.

Swordfish

In phenoxyethanol application, induction phase times of swordtail fish were shown in Figure 3A and total induction times were shown in Figure 3B. Similarly, recovery phase times were shown in Figure 4A and total recovery times were shown in Figure 4B.

In the assessment, a difference between the total induction times of female and male individuals in 2-phenoxiethanol dose was found ($p<0.05$). A similar situation was also found for male individuals ($p<0.05$). Also, when each dose was assessed one by one, a difference was not determined between the induction times of female and male individuals ($p>0.05$).

When the recovery times of the fish exposed to phenoxyethanol were assessed, it was found that there was a difference in total recovery times of female and male individuals in themselves in all concentrations/ doses ($p<0.05$). Besides, variability in female and male individuals' recovery among doses was identified. Differences were found in all application doses ($p<0.05$). In clove-oil applications, induction times of swordtail fish were shown in Figure 3A and total recovery times were shown in Figure 3B. Similarly, recovery times were shown in Figure 4A and total recovery times were shown in Figure 4B.

In the application carried out with clove oil, a difference between female and male individuals' induction times among doses was found ($p<0.05$). Together with this, when each dose was assessed one by one, it was found that there was a difference between induction times of male and female individuals in 2 and 6 ml/L dose/ concentrations ($p<0.05$).

When recovery times of the fish were assessed, a difference between female and male individuals for all doses was found ($p<0.05$). In 4 ml/L. clove oil application, there was not a difference between the recovery times of female and male individuals ($p>0.05$), yet in other application doses, differences were identified ($p<0.05$).

DISCUSSION

When fish are exposed to long-term stress in aquaculture environments, they are affected adversely (Roos and Roos, 2008). For this reason, anesthetic substances have been a subject of study for many marine and freshwater fish species. As a result of the studies, it was found that if inappropriate dose of anesthetics were applied, death or stress-based problems might be encountered due to being exposed to long-term or the inappropriate dose (Feng *et al*., 2011; Hasan *et al*., 2013). Abiotic factors, biotic factors, anesthetic choice and application conditions should be taken into consideration when fish are induced (Iversen *et al*., 2003).

There are many studies which try to determine the effective dose and effects on feeding (Rabbitfish *Siganus lineatus* Soto and Burhan-ud-Din, 1995) in different fish species. In this study, after two hours of clove oil application, clownfish fish and swordfish were fed successfully; however, after phenoxyethanol application the fish were fed the following day in any case.

In the studies carried out with phenoxyethanol; the shorter induction time and the longer recovery time were observed (rabbitfish, *Siganus rivulatus,* Soto and Burhan-ud-Din, 1995; marbled spinefoot, Ghanawi *et al*., 2013; Rainbow Trout *Oncorhynchus mykiss*, Yıldız *et al*., 2013). Furthermore, as the dose increased in Senegalese sole (Weber *et al*., 2009), the time of inductions and the time of recovery lessened. In this study, in all dose of phenoxyethanol induction was under 150 seconds and the more the dose was, the shorter induction time was. In recovery, recovery realized in all dose/concentrations under 300 seconds in total time and as dose/concentration increased, recovery time increased.

In our study, it was found that as the dose/concentration was increased in clove oil application, the time of induction decreased. The similar situation was also seen in rabbit fish (*Siganus rivulatus*) (Soto and Burhan-ud-Din, 1995; Ghanawi *et al*., 2013), Rainbow Trout (*Oncorhynchus mykiss*) (Yıldız *et al*., 2013) species. It was observed that the recovery from anaesthesia of fish treated with clove oil was longer than the other anestesic (rabbitfish. *Siganus rivulatus* (Soto and Burhan-ud-Din, 1995; Keene *et al*., 1998) in juvenile Russian sturgeon, Mylonasa *et al*. (2005) in European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*), Munday and Wilson (1997) in coral reef fish (*Pomacentrus amboiensis*) and Feng *et al*. (2011) in juvenile Siberian sturgeon; rainbow trout, *Oncorhynchus mykiss* (Yıldız *et al*. (2013); channel catfish, *Ictalurus punctatus* (Waterstrat, 1999). Besides that, there was an inverse proportional dependence between recovery time and clove oil concentration in rabbitfish (*Siganus rivulatus*) Ghanawi *et al*. (2013) and in pike (*Esox lucius* L.) Zaikov *et al*. (2008). In *Senegalese solea* (Weber *et al*., 2009) the time of recovery was independent of the amount of dose. According to Mylonasa *et al*. (2005), because the fish exposed to high amounts of dose induced rapidly, they were taken to recovery phase without being kept in anesthetics for long. The fact that recovery time takes long in fish which are applied anesthetic may be an advantage during the manual intervention of many fish (Woody *et al*. 2002). Furthermore, lessening fish stress decreases oxygen consumption because it decreases the metabolic rate and swimming speed during transportation (Chandroo *et al*., 2005). As a result in short-term applications on clownfish, the ideal amount of anesthetic substance might be 0.4 ml/L in phenoxyethanol, and 1 ml/L in clove oil. As a result for swordfish, it was clearly seen that both anesthetic substances could be used for both female and male individuals of swordtail fish. When the induction and recovery times of both substances were considered in terms of usability in female and male individuals of swordtail fish, there were no big differences. In this context, it was found out that in ideal anesthetic substance classification, phenoxyethanol dose in which the time of induction was below 3 min and the timing recovery was below 5 min was 1 ml/L in short-term applications in swordtail fish. The rate for clove oil was found to be 2 ml/L. The fact that recovery took long in anesthetized fish might be seen as an advantage in manual intervention (Woody *et al*. 2002). Furthermore, decreasing the stress of fish decreases the oxygen consumption because it decreases the metabolic rate and swimming speed (Chandroo *et al*., 2005).

CONCLUSION

The suggested dose of clove oil in this study was 6 ml/L for female and male fish. Similarly, the dose of phenoxy ethanol was 5 ml/L. It is apparent that when the economic criteria in anesthetic substance classification are considered, clove oil is more advantageous than phenoxyethanol. In terms of environmental interaction, clove oil should be important for aquaculture.

Statement of conflict of interest

Authors have declared no conflict of interest.

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