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Potential anesthetic properties of Bay Leaf (*Laurus nobilis*) essential oil compared with 2-Phenoxyethanol on Blue Dolphin Cichlid, *Cyrtocara moorii*

Volkan KIZAK[1]*, Erkan CAN[2], Şafak SEYHANEYILDIZ CAN[3]

[1]Fisheries Faculty, Department of Aquaculture, Munzur University, Tunceli, Turkey
[2]Fisheries Faculty, Department of Aquaculture, Katip Celebi University, Izmir, Turkey
[3]Engineering Faculty, Department of Bioengineering, Munzur University, Tunceli, Turkey

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Abstract

The efficacy of Bay leaf (*Laurus nobilis*) essential oil (LnEO) as a herbal anesthetic agent was evaluated with 2-Phenoxyethanol (2-PE) in Blue Dolphin Cichlid, *Cyrtocara moorii*. Fish were exposed to different anesthetic concentrations and the lowest effective concentrations (LECs) were determined according to deep anesthesia (AD<3 min) and full recovery (RF<5 min) times. LnEO showed anesthetic properties, and all applied concentrations of both agents induced AD in *C. moorii*. The LECs of LnEO and 2-PE for *C. moorii* were determined as 800 µl L-1 (AD; RF → 183 ± 6.3 s; 191 ± 2.6 s) and 600 µl L-1 (AD; RF → 171 ± 3.2 s; 239 ± 4.9 s), respectively. Significant differences were found between anesthetic agents in terms of deep anesthesia and full recovery times for the same concentrations (p<0.05). Induction and recovery times decreased with increasing in LnEO concentrations. On the other hand, anesthesia times were decreased with increasing of 2-PE concentrations, while recovery times increased. Induction and recovery times for LnEO were significantly dependent on concentrations and positive relationships were recorded between AD and RF, whereas the relationship was negative in 2-PE. Bay leaf essential oil has not been used as an anesthetic agent in fish until now. Our results showed that LnEO was an effective anesthetic agent with some minor side effects on *C. moorii*.

* Corresponding author. Tel: +905438991902, email: v.kizak@gmail.com, volkan.kizak@munzur.edu.tr

Introduction

Handling procedures such as morphometric measurements, grading, or transporting operations can cause stress that influences fish behavior and physiology negatively (Ross and Ross, 2008). These routine operations in aquaculture often induce a physiological stress response, which may result in immunosuppression and growth retardation on fish (Roubach et al., 2005; Heo and Shin, 2010). Anesthetics may reduce stress-induced damage to the fish, and also decrease the physiological response to stress (Weber et al., 2009; Pawar et al., 2011). Anesthesia is an ideal option to minimize the negative impact of handling on fish and carried out at this point for eliminating the adverse effects of stress (Kizak et al., 2018b)

MS-222, 2-Phenoxyethanol and clove oil anesthetics are commonly used in aquaculture. An ideal anesthetic agent should be harmless to the user, fish and environment (Kizak et al., 2013). Several chemical anesthetic agents have some side effects on fish and humans (Velisek et al., 2007; Zahl et al., 2012; Fernández-Parra et al., 2017). Bressler and Ron (2004) reported that the use of clove oil as an anesthetic agent is preferred to the use of benzocaine because it does not pose a chemical health hazard to people who handle the fish or to consumers nor does it cause any significant immunodepression effect on the fish. In recent years, herbal essential oils used as natural anesthetics in aquaculture seem to be more beneficial in terms of fish welfare, environment and human health than synthetic anesthetic agents.

Many herbal essential oils such as *Lippia alba*, *Hesperozygis ringens*, *Lippia sidoides*, *Ocotea acutifolia*, *Aloysia triphylla*, *Cymbopogon flexuosus*, *Matricaria chamomilla*, *Melaleuca alternifolia*, *Pelargonium graveolens*, *Aniba rosaedora, Cymbopogon citratus, Coriandrum sativum, Bursera delpechiana and Lavandula hybrida* have been tested as potential anesthetic agents on aquatic species in last decade (Cunha et al., 2010; Silva et al., 2013; Santos et al., 2017; Can et al., 2017; Correia et al., 2018; Can et al., 2018; Kizak et al., 2018a; Kizak et al., 2018b; Can et al., 2019).
Laurus nobilis (Lauraceae) commonly known as bay leaf, growing up to ten meters high widespread in the Mediterranean area, and widely cultivated in many countries with moderate and subtropical climate (Di Leo Lira et al., 2009). The biologically active compounds of *L. nobilis* essential oils (**Table 1**) traded as a spice and medicinal items were methyl eugenol and 1,8-cineole have pharmacological properties, muscle relaxant effect, and anti-inflammatory activity, respectively (Peris and Blázquez, 2015).

Table 1 Main components of *Laurus nobilis* essential oil (LnEO).

Component	*Percentages (%)*
1.8-Cineole	53.30
Sabinene	9.96
Alpha terpinenyl acetate	9.13
Alpha pinene	4.41
Alpha terpineol	3.37
Terpinene-4-ol	2.61
Limonene	1.60
Gamma terpinene	1.24
Cymene	1.23
Linalool	1.06
Terpinen-4-yl acetate	0.83
Beta myrcene	0.79
Methyl eugenol	0.65
Beta caryophyllene	0.64
Alpha terpinene	0.58
Beta elemene	0.51
Eugenol	0.42
Alpha thujene	0.39
Alpha phellandrene	0.32
Cineole	0.29
Others	6.67
TOTAL	100

(Source: Nu-Ka Defne Essencia)

Ornamental fishes are commercially valuable living beings for aquarists and trade (Kizak et al., 2018b), which has grown significantly in the last 40 years. *Cyrtocara moorii* is a species of cichlid endemic to Lake Malawi and is popular among aquarium hobbyists, where it is generally known as blue dolphin cichlid (Can and Sümer, 2019).
Bay leaf essential oil (LnEO) has not been used as an anesthetic agent in fish until now. This study aims to investigate the efficacy of LnEO as a potential herbal anesthetic agent on Blue Dolphin Cichlid.

Material and Methods

Experimental fish and water quality. Experiments were conducted on Blue Dolphin Cichlid, *Cyrtocara moorii* (mean body weight 9.89 ± 0.45 g) (n=91). The fish were purchased from a private pet-shop in Elazig (Turkey) and were transferred to the Laboratory of Bioengineering Department, Munzur University (Turkey). They were stocked into four glass aquaria (each one 100 L) separately and acclimatized to water gradually. Blue Dolphin Cichlids were fed ad libitum once daily, approximately 2% of their own weight with commercial granulate feed (Sera Granured, Germany) containing 42.5% crude protein and starved 24 h prior to starting of the experiments. Water quality parameters were about 23.4±0.1°C, pH 7.84, and DO 7.42±0.13 mg L-1 during the experiment.
Anesthetic agent. Bay leaf (*Laurus nobilis*) essential oil (LnEO) was investigated as a potential herbal anesthetic agent in this study. LnEO was purchased from a commercial company (Nu-Ka Defne Essencia, Turkey), which also provided the chemical compositions of LnEO. The main components of LnEO were given in **Table 1**. 2-Phenoxyethanol (2-PE) (Sigma-Aldrich) was used as a chemical anesthetic agent.

LnEO was dissolved in ethanol (94% purity) to enable better dissolution in water during treatments. Preparation of essential oil concentration was performed according to the method of Can et al. (2018), where the oil was poured into a 15 ml plastic capped tube at the desired amount and then ten-fold ethanol was added to each tube. After shaking, 10 ml of treatment water was added into essential oil + ethanol solution and shaken again. Then, the solution was poured into a glass aquarium filled with 2 L of treatment water. 2-PE was directly dissolved in treatment water.

Experimental design
Initially, preliminary tests were conducted to find out the lowest anesthetic concentration of anesthetic agents for *C. moorii*, which did not reach to deep anesthesia at 200 µl L-1 and 300 µl L-1 with LnEO and 2-PE, respectively. After that, fish were exposed to different concentrations of the LnEO (300, 400, 500, 600, 700 and 800 µl L-1) and 2-PE (400, 500, 600, 700, 800 and 900 µl L-1) to determine the optimal induction and recovery times. Determination of anesthesia (A) and recovery (R) stages are shown in **Table 2**. Each fish was assessed individually and used only once in each replicate at defined concentrations. Seven fish were applied for each concentration to find the lowest effective concentration (LEC) that were chosen according to Kizak et al. (2018a): Deep anesthesia (AD) should be achieved in <3 min and the full recovery (RF) time should not be exceeded 5 min. Induction and recovery times of different concentrations for LnEO and 2-PE were recorded by a digital stopwatch. Fish were netted from the holding tank, transferred individually into an anesthesia glass aquarium (2 L) and observed for equilibrium, opercular movements and response to tail pinch. When they lost total equilibrium and reached the AD stage, the fish were removed by net, weighed and transferred recovery glass aquaria (2 L) that contains anesthesia-free water to assess recovery time. Once recovered, fish were transferred into a stock tank and monitored for survival and abnormal behavior following 24 hours. Additionally, a solution with 8 ml L-1 of ethanol was tested in seven fish within the maximum exposure time (3 min) to examine the efficacy of ethanol (Can et al., 2017).

Table 2 Anesthesia stages and description of fish behaviour (A: Anesthesia, R: Recovery) (Mylonas et al., 2005; Kizak et al., 2018a).

Stages of A and R	*Description of Fish Behaviour*
A_I (Initial Induction)	Total loss of equilibrium, slow but regular opercular rate
A_D (Deep Anesthesia)	No reflex, opercular movements slow and irregular, no respond to strong external stimulus
R_I (Initial Recovery)	Total recovery of equilibrium, swimming erratic
R_F (Full Recovery)	Total behavioural recovery, normal swimming

Statistical analysis. Normality and homogeneity of data were checked to comply with the assumptions of ANOVA. One-way ANOVA, followed by the Duncan Multiple Range Test, was used to determine significant differences among means. Results are presented as means ± SE and statistically significant differences are expressed as $p<0.05$. All statistical analyzes were carried out using SPSS (Version 20.00). Regression equations, performed with Excel, showed a relationship between anesthetic concentrations and induction/recovery times as well as between induction and recovery times.

Discussion

In the present study, Blue Dolphin Cichlid, *Cyrtocara moorii* (*C. moorii*), was exposed to different concentrations of *Laurus nobilis* essential oil (LnEO) and 2-Phenoxyethanol (2-PE). No reports were found on the efficacy of the anesthetic of LnEO on fish. Our results showed that LnEO was an effective anesthetic agent with some minor side effects on *C. moorii*.

L. nobilis (Lauraceae) yields an essential oil (LnEO) with a high content of 1,8-Cineole (Peris and Blázquez, 2015). In the present study, the main components of LnEO were 1,8-Cineole (53.30 %), Sabinene (9.96 %), Alpha terpinenyl acetate (9.13 %) Alpha pinene (4.41%) and Alpha terpineol (3.37%) (**Table 1**). Mazandarani and Hoseini (2017) and Mirghaed et al. (2018) reported that 1,8-Cineole induced deep anesthesia in common carp and rainbow trout. These results showed that the component 1,8-Cineole has anesthetic properties on fish species. In the present study, LnEO is expected to have an anesthetic effect on *C. moorii* because of its main component, 1,8-Cineole.

Stress and injury due to handling procedures in aquaculture can be prevented or decreased by anesthesia. In the anesthetic procedure, induction and recovery of fish within a certain time range is a necessity. Induction should occur in less than 3 min, but the recovery time should not be longer than 5 min (Ross and Ross, 2008). This time range for induction and recovery of fish is sufficient to carry out routine aquacultural operations (Weber et al., 2009). LnEO was found to be an effective anesthetic for *C. moorii*. Increased concentrations lead to faster induction in both anesthetic agents, whereas the recovery times vary. Recovery times decreased with increasing of LnEO concentrations, while prolonged recovery times were recorded for 2-PE (**Table 3, Figure 1**). Deep anesthesia was achieved in all applied concentrations of LnEO and 2-PE in *C. moorii*. According to the aforementioned criteria, the LECs of LnEO and 2-PE were determined as 800 µl L-1 and 600 µl L-1, respectively. 1,8-Cineole, which is the main component of LnEO, was reported as a potential anesthetic agent for common carp and rainbow trout (Mazandarani and Hoseini, 2017; Mirghaed et al., 2018). According to these publications, 1,8-Cineole anesthetize common carp at 595 µl L-1 within 3 min and is efficient to anesthetize trout at a concentration of 200–800 µl L-1. Differences in effective concentrations of essential oil can be explained by species and age differences of fishes (Kizak et al., 2018b) and the activity of the biological compound.

Table 3. The anesthesia stage times at different concentrations of LnEO and 2-PE for *C. moorii*.

LnEO

Concentration	*Induction Time (s)*		*Recovery Time (s)*	
µl L⁻¹	A_I	A_D	R_I	R_F
300	258 ± 12.6^{l}	395 ± 10.9^{i}	167 ± 12.1^{de}	256 ± 9.9^{g}
400	218 ± 9.8^{k}	329 ± 5.7^{g}	157 ± 5.6^{d}	251 ± 10.5^{fg}
500	201 ± 1.5^{j}	296 ± 8.2^{f}	186 ± 6.6^{f}	249 ± 7.7^{fg}
600	180 ± 2.0^{i}	244 ± 6.2^{e}	154 ± 5.9^{d}	204 ± 2.8^{d}
700	161 ± 4.7^{h}	250 ± 9.3^{e}	171 ± 1.1^{e}	206 ± 3.1^{d}
800	91 ± 1.3^{e}	183 ± 6.3^{d}	138 ± 3.3^{c}	191 ± 2.6^{c}

2-PE

Concentration	*Induction Time (s)*		*Recovery Time (s)*	
µl L⁻¹	A_I	A_D	R_I	R_F
400	119 ± 3.1^{g}	365 ± 6.4^{h}	124 ± 1.5^{b}	161 ± 3.1^{a}
500	111 ± 4.8^{f}	187 ± 5.9^{d}	108 ± 1.9^{a}	175 ± 3.8^{b}
600	70 ± 4.3^{d}	171 ± 3.2^{c}	198 ± 2.7^{g}	239 ± 4.9^{ef}
700	50 ± 3.2^{c}	100 ± 1.7^{b}	201 ± 2.0^{g}	232 ± 2.3^{e}
800	40 ± 1.9^{b}	98 ± 1.7^{b}	241 ± 3.7^{h}	253 ± 2.5^{g}
900	36 ± 1.8^{a}	60 ± 1.5^{a}	243 ± 3.4^{h}	291 ± 6.0^{h}

*Values (mean±SE) with different superscripts within the same column are significantly different ($p<0.05$).

Generally, the recovery from anesthesia is dependent on the concentration of the anesthetic (Weyl et al., 1996). Recovery times in LnEO and 2-PE were negatively and positively correlated with anesthetic concentrations, respectively (**Figure 1**). From this point, the relationship between induction and recovery times are positive in LnEO, whereas this relationship is negative in 2-PE (**Figure 2**). Sena et al. (2016) found that the increased anesthetic concentration of *L. alba* essential oil resulted in a decrease in the times of induction and recovery. Limma-Netto et al. (2016) also reported that exposure of *C. flexuosus* essential oil resulted in a reduction of recovery time. On the other hand, a negative relationship between recovery and induction times were stated in several studies (Weyl et al., 1996; Can et al., 2018; Kizak et al., 2018a). Applied anesthetic agents, water temperature and species of fish can affect the recovery times (Mylonas et al., 2005; Limma-Netto et al., 2016).

Some previous studies showed that the component 1,8-Cineole could cause several side effects such as tail-up swimming and failed to completely muscle tone in common carp and slightly lower side effects in rainbow trout (Mazandarani and Hoseini, 2017; Mirghaed et al., 2018). In the present study, LnEO exposure did not cause mortality, but some minor side effects such as coughing, interruption of ventilatory for a while and appearing of black bands on lateral sides were observed during the treatments. Rezende et al. (2017) stated that eucalyptus, mint, and clove essential oils impede movement in Nile tilapia, but also cause behavioral changes in skin color and pattern, and changes in swimming pattern. Skin color and pattern with alternating vertical bands of dark and light shades indicate the stress in fish (Van der Salm et al., 2006; Rezende et al., 2017).

In conclusion, despite leading to minor side effects, *Laurus nobilis* essential oil showed mostly positive anesthetic properties on Blue Dolphin Cichlid, *Cyrtocara moorii*. It provides almost fast anesthesia and recovery process as well as 2-Phenoxyethanol. These properties can make it being a potential herbal anesthetic for aquacultural handling procedures. Further studies are necessary to determine the physiological effects of LnEO on fish.

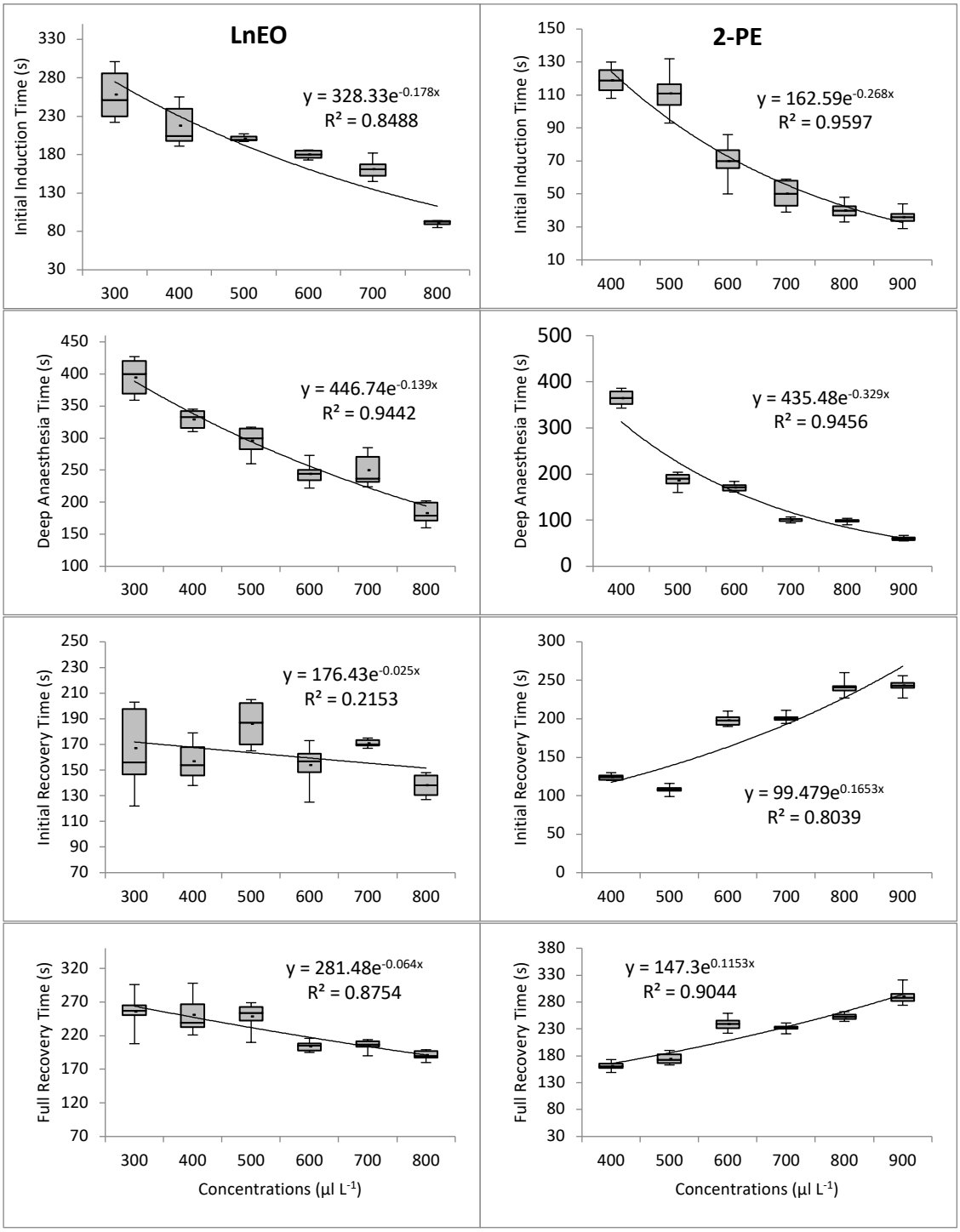

Figure 1 Box plots of time (s) to induction (A_I and A_D) and recovery (R_I and R_F) distributions for *C. moorii* anesthetized with various concentrations of LnEO and 2-PE. Relationships between induction/recovery stage times and and anesthetic agents for *C. moorii*.

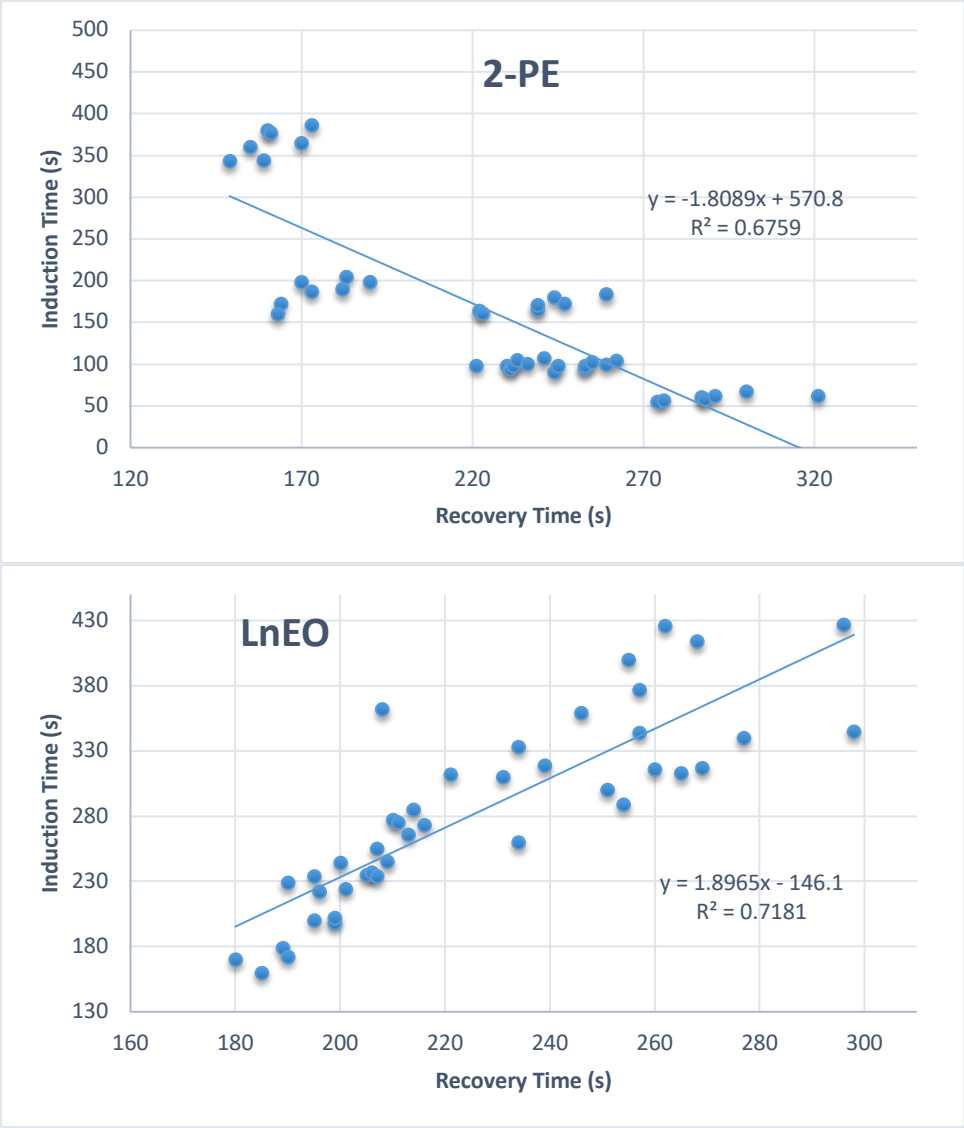

Figure 2 Relationship between deep anesthesia (A_D) and full recovery (R_F) times in *C. moorii* anesthetized by various concentrations of LnEO and 2-PE.

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