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SHORT PAPER

The Efficacy, Physiological Responses and Hematology of Persian Sturgeon, *Acipenser persicus*, to Clove Oil as an Anesthetic Agent

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Abstract

In order to examine the efficacy of clove oil as an anesthetic on Persian sturgeon, *Acipenser persicus*, we conducted an experiment. We also investigate if clove oil anesthesia suppressed the normal plasma cortisol and glucose level increase in this species or not. The Effects of clove oil on Hematological factors (Hb, Hct, and WBC) was examined as well. Three different concentrations of clove oil (200, 300, 400 mg/L) combined the two water temperature (20 and 24°C) were assessed. The combination of 400 mg/L and 24°C was the best treatment for anesthetizing as well as recovery. From one trial (concentration: 300 mg/L and temperature: 24°C), blood samples were taken on 0 (immediately upon placement in recovery tank), 1, 6, 24 and 72 hours post recovery in order to evaluate physiological responses. After 72 h both blood glucose and cortisol reach the level similar to the control group which was before applying clove oil. Hematological parameters after some fluctuation reached its level the same as control group of the experiments.

Keywords: Aquaculture, anesthesia, fish, physiology, sturgeon, *Acipenser persicus*.

İran Mersin Balığının, *Acipenser persicus*, Anestezik Ajan Olarak Karanfil Yağına, Efikasitesi, Fizyolojik Tepkileri ve Hematolojisi

Özet

İran kökenli mersin balığında *Acipenser persicus* anestezik olarak karanfil yağının kullanılabilirliğini değerlendirmek amacıyla deneme yapılmıştır. Aynı balık türünde karanfil yağının normal plazma kortizonunu ve glikoz seviyesini değiştirmesi ve karanfil yağının hematolojik faktörlere (Hb, Hct, and WBC) olan etkisi de araştırılmıştır. İki su sıcaklığına (20 ve 24°C) karşın üç farklı karanfil yağı dozu (200, 300, 400 mg/L) kullanılmıştır. 400 mg/L ve 24°C kombinasyonu hem anestezi hem de uyanma için en iyi muamele olarak değerlendirilmiştir. 300 mg/L ve 24°C kombinasyonundaki balıklardan ayılmalarından sonraki 1, 6, 24 ve 72. saatlerde, fizyolojik tepkilerini değerlendirmek amacıyla kan örnekleri alınmıştır. 72. saatin sonunda hem kan glikoz seviyesi hem de kortizon seviyeleri kontrol grubuyla aynı seviyelere ulaşmıştır. Hematolojik parametreler hafif dalgalanmaların ardından kontrol grubuyla aynı seviyelere ulaşmıştır.

Anahtar Kelimeler: Yetiştiricilik, anestezi, balık fizyolojisi, mersin balığı, *Acipenser persicus*.

Introduction

In fisheries and aquaculture, anesthetics are helpful for reducing the stress caused by handling (Small, 2003), transportation (Bosworth *et al.*, 2007), Artificial insemination (McGovern-Hopkins *et al.*, 2003), sampling (Ackerman and Bellwood, 2002; Baker *et al.*, 2005), tagging (Davenport *et al.*, 2002) and surgical preparations for physiological investigations (Warren *et al.*, 2004).

Choosing an anesthetic attributes to several characteristics including its efficacy, availability; cost-effectiveness; ease of use; nature of the study; and safety for the user including fish, humans and the environment (Iversen *et al.*, 2003; Mylonas *et al.*, 2005).

Since fish breathe through gills rather than lungs, anesthetic agents are greatly inhaled with gills. As a result, anesthesia must be added to the tank water and delivered through an aquatic medium (Schaeffer, 1997). Therefore, the relationship between the epithelium surface of the gill and the body volume as well as thickness of epithelium affect the efficacy of anesthetics (Ross and Ross, 1983; Davenport, 2002). The Other Biological factors, including species, the stage of life cycle and age, size and weight, lipid content, body condition and disease status also influence the metabolic rate and therefore

the pharmacokinetics of the anesthetic compound (Iversen *et al.*, 2003).

Water condition, such as temperature and pH can also affect the efficacy of an anesthetic solution on species (Coyle *et al.*, 2004; Ackerman *et al.*, 2005).

Eugenol, clove active ingredients, has been widely tested for human consumption and is listed as substances generally regarded as safe (GRAS) in humans at levels less than 1500 mg/L. (Anderson *et al.*, 1997; Keene *et al.*, 1998; Iversen *et al.*, 2003).

Clove oil, natural oil derived from the clove plant, *Eugenia caryophyllata*, has some of the characteristics considered for an ideal anesthetic agent. The active ingredient of clove oil is eugenol (4-allyl-2-methoxyphenol), a phenolic compound (Sladky, 2001; Iversen *et al.*, 2003), which inhibit the prostaglandin H synthase (PHS) and result in analgesic effects of clove oil (Keene *et al.*, 1998) What's more, the eugenol-based anesthetics are effective at low dosages, inexpensive, easily obtained and capable to reduce stress. They are also organic substances safe for both environment and user (Iversen *et al.*, 2003; McGovern-Hopkins *et al.*, 2003). Therefore, it could be a promising anesthetic agent in aquaculture and many studies have done to evaluate its efficacy on some species (Anderson *et al.*, 1997; Munday and Wilson, 1997; Keene *et al.*, 1998; Sladky *et al.*, 2001; Iversen *et al.*, 2003; Small, 2003; Cooke *et al.*, 2004; Davis and Griffin, 2004; Mylonas *et al.*, 2005; Ribas *et al.*, 2007).

Clove oil in Iran is used as an effective agents for anesthetizing fish in aquaculture facilities in order to mitigate the handling stress due to the grading, transporting and artificial spawning (Mehrabi, 2000).

Persian sturgeon is an endangered species (Bagheri *et al.*, 2008). It is spawned artificially in aquaculture facilities with the aim of restocking to improve its population. So some studies were done on effects of anesthetic agents on brood stocks and fries. Raising sturgeons for producing broodstocks in order to reduce dependency on natural populations is very promising (Hedayati *et al.*, 2008). During culturing and breeding practices, stressful functions such as handling and transportation might affect its survival and growth, so using anesthetic agents could be helpful (Cataldi *et al.*, 1998).

In order to examine the efficacy of clove oil as an anesthetic on Persian sturgeon, *Acipenser persicus*, we conducted an experiment. We also investigate to determine whether clove oil anesthesia suppressed the normal plasma cortisol and glucose elevating in this species or not. Hematological factors which could be affected by anesthetic agent were analyzed as well.

Materials and Methods

Fish and Rearing Condition

The experiments conducted on juvenile Persian sturgeon (average weight: 148.7±22.6 g) produced at the Institute of Aquaculture of the Marjani for Sturgeon, Golestan, Iran. Prior to the study, fish were maintained in groups in 150 L aquariums in an indoor facility; fish had been maintained in this facility for more than 2 years. For the purpose of the study, fish were housed separately in experimental aquaria and acclimated to it for a minimum of 2 weeks. The aquaria shared a common source of water with a steady temperature of 24°C. Throughout the acclimatization period and during the experiment; environmental conditions were monitored and maintained within a narrow range of variable. Fish were kept under natural photoperiodic conditions, fed on hand with commercially formulated pellet and fasted for 24 h prior to each experiment.

Anesthesia Preparation and Experiment

First part of the experiments was examining anesthetic effects on fish. Before beginning the study, we conducted a pilot one and found that the characteristics defined the induction of different stages of anesthesia and recovery (Table 1) is similar to those reported previously by Iwama *et al.* (1989). We chose three different concentrations of clove oil (200, 300, 400 mg/L) according to the before scientific papers and previously pilot study. For preparation the desired dose of clove oil, we made up a 10% stock solution (1 ml clove oil +9 ml of maintaining water). To make a 200 mg/L solution, we took 2 ml of the stock solution and mix it with one liter of water and this procedure applied for the other two concentrations (McGovern-Hopkins *et al.*, 2003; Ribas, 2007).

Since many aquaculturists and clinicians add anesthetic agents directly to water baths to achieve the desired dose (Sladky, 2001), we applied prepared clove oil solution into water.

As far as diseased or weakened animals are much more susceptible to anesthetic treatment (Coyle *et al.*, 2004), six healthy Fish were anesthetized by immersing them in a bath containing anesthetic agent so that it is absorbed through the gills and rapidly enters the blood stream. For examination the effects of temperature, we combined the water temperature (20 and 24°C) and anesthesia concentrations. The fishes were placed through knotless dip net in an aerated container containing the holding tank water (Schaeffer, 1997). Aeration provided extra oxygen required during induction which causes increased respiration. To prevent abrading the skin of the fish, the handler wore wet latex gloves and gently transferred the fish into the container. When an anesthetic is first administered (induction), fish may go through an excitement phase, as inhibitory neurons are depressed before achieving anesthesia and becoming hyperactive for a few seconds. (Sladky *et al.*, 2001; Coyle *et al.*, 2004), so a glass cover on the induction tank was used to make the anesthetic stages

visible for the operator.

For the anesthetic effect, a video cassette recorder (DSC-W80, Japan) was used to record fish behaviour for subsequent analyses (Cooke *et al.*, 2004). Two observers made decision using the Table 1, according to the Iwama *et al.* (1989).

Immediately after fish in each trial reach stage III, on a wet towel were weighed; total length was recorded and placed individually within 1 min into a recovery aquarium.

The recovery tank used the same water as anesthetic bath (at a similar temperature and chemistry) supplied with flow-through water at a high exchange rate to ensure that fish were always in contact with clean water. Water quality was carefully controlled during the experiment.

Anesthetic and Recovery times to stage A3 and R3, respectively, were recorded from the time placing the fish in Anesthetic and recovery tank to the nearest second using an electronic stop-watch (Mylonas *et al.*, 2005).

Physiological Experiment

For the second part of the experiment, from one trial (concentration: 300 mg/L and temperature: 24°C), apart from anesthetic experiment, five individual (average weight±sd: 146.8±17.3 g) netted and bled serially at the times: 0 (immediately upon placement in recovery tank), 1, 6, 24 and 72 hours post recovery. Before using anesthesia, a group of fish bled as a control (C). For physiological responses, Blood (4 ml) was collected within 2 min of the fish being captured from the caudal vasculature using a syringe (Webb *et al.*, 2007). The blood divided into two aliquots, one part was transferred to a 2 ml vacationer tube containing heparin sodium, shook for 2 minutes gently and stored in refrigerator prior to hematological analysis. Oxygen transport characteristics (hematocrit, hemoglobin) as well as White Blood Cells (WBC) were analysed with. (Baker *et al*., 2005)

The other part of aliquots transferred into a 1.5 ml microcentrifuge tubes and centrifuged for 15 minutes (Fast *et al.*, 2008) at 4°C. The plasma removed and transferred to another 1.5 ml microtube and stored frozen at −70°C until subsequent analysis for metabolite concentration, cortisol and glucose (Baker *et al*., 2005).

Results

A summary of the average time to Anesthetic stages at each of the tested dosages associated with the water temperature is presented in Table 2. Response time at tested dosages along with water temperature was rapidly occurred in less than 1.5 minute after exposure to the clove oil. All experimental fish were successfully revived and no mortalities observed by 72 h post-treatment.

Mean plasma cortisol concentration was 17.88±1.3 mg/ml for the control group; this was before the beginning of the experiment. Upon transporting anesthetic fish into recovery tank, plasma cortisol concentration upgraded to 121.58±14.85 mg/ml. Although after 72 h decreasing in mean plasma cortisol concentration was revealed, it did not reach the similar concentration to that of the control.

Table1. Stages of Anesthesia and Recovery

Stages of Anesthesia	Description
I	Loss of equilibrium
II	Loss of gross body movements but with continued opercular movements'
III	As in stage II with cessation of opercular movements
Stages of Recovery	**Description**
I	Body immobilized but opercular movements just starting
II	Regular opercular movements and gross body movements beginning
III	Equilibrium regained and preanesthetic appearance

From Iwana *et al*. (1989), modified by Ackerman *et al*. (2005)

Table2. Effects of clove oil and water temperature on anaesthesia and recovery of Persian sturgeon

Dose(mg/L)	Anesthetic time(s)			Recovery time(s)			
	20°C		24°C	20°C			24°C
200	168±51.5		136±38.5	181±60.2			144±66.86
300	158±29.5		122±19.5	217±96.4			140±9.5
400	121±28.9		110±30.2	223±33.79			159±23.21
Two-way ANOVA							
	DF	Mean square	*F*-value	*p*-value	DF	Mean square	*F*-value	*P*-value
Dose	2	2784.92	2.35	0.12	2	1645.24	0.52	0.61
Temperature	1	4118.64	3.47	0.79	1	21312.96	0.67	0.01
Interaction	2	354.79	0.29	0.74	2	835.84	0.26	0.77

[1] Each value is mean±standard deviation (n=6). Values are not significantly different (P<0.05)

However, mean plasma cortisol concentration were not significantly (P<0.05) different between 72 h and control group (Figure1).

Plasma glucose concentration was 46.6±4.16 mg/dl in control group and elevated upon placement in recovery tank. Nevertheless, elevating plasma glucose concentration stopped and alleviating started until 72 h which was no significantly (P<0.05) different among control, 24 h and 72 h (Figure 2).

Oxygen transport characteristics in bloods as well as WBC analyzing are demonstrated in Figure 3, 4 and 5.

Discussion

It was shown that the Persian sturgeon exposed to the clove oil tested concentrations in our experiment; get in anesthetic phase less than 2.1 minutes equal to 2.8 minutes. Recovery for all treatments was less than 223 seconds equal to 3.7 minutes. The anesthetic should be easy to administer, effective at low doses and reasonable in cost (Davenport *et al.*, 2002; Coyle *et al.*, 2004; Mylonas *et al.*, 2005).

Regarding the efficacy criteria for an ideal anesthetic agent to be suitable for a researcher, it should induce anesthesia rapidly with minimum hyperactivity or stress, produce anesthesia within 3 min or less. When the animal is removed from the anesthetic and placed in recovery tank containing clean water, recovery should be rapid, within 5 min or less. Therefore, the tested clove oil concentrations are

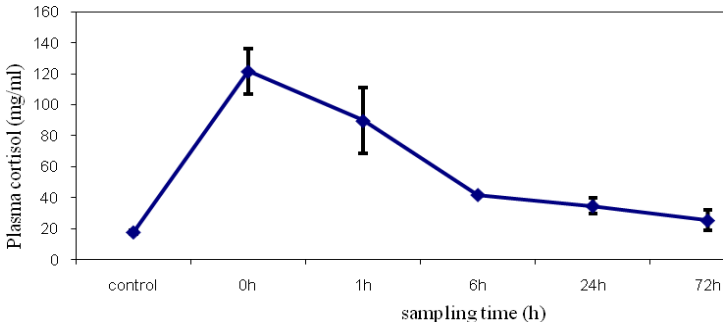

Figure 1. Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma cortisol on the bars indicate statistical significance between treatment and control groups at a sampling time.

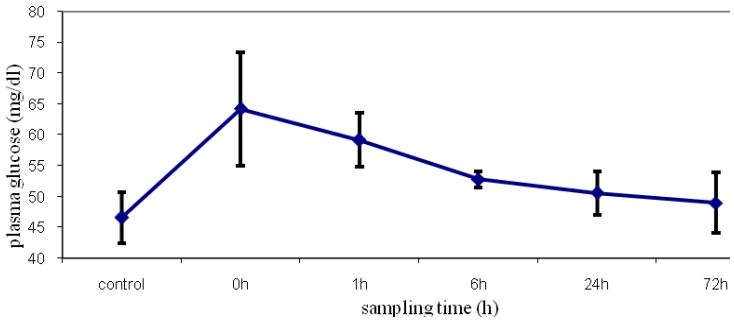

Figure 2. Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma glucose on the bars indicate statistical significance between treatment and control groups at a sampling time.

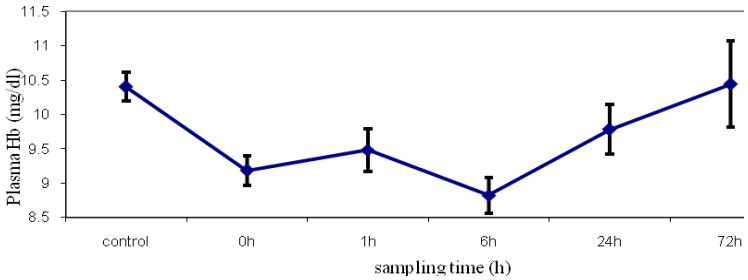

Figure 3. Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma hemoglobin on the bars indicate statistical significance between treatment and control groups at a sampling time.

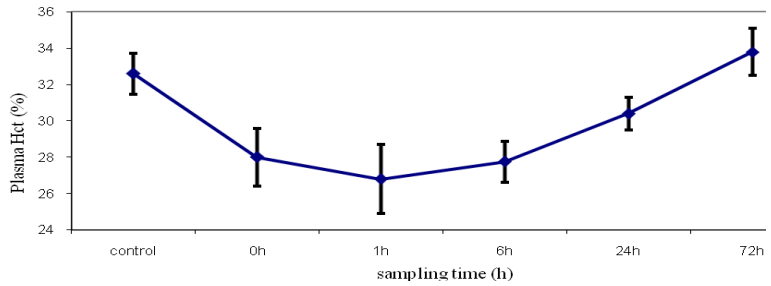

Figure 4. Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma haematocrite on the bars indicate statistical significance between treatment and control groups at a sampling time.

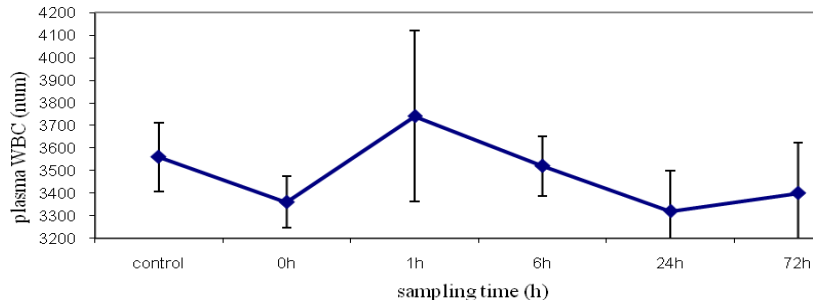

Figure 5. Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma white blood cells on the bars indicate statistical significance between treatment and control groups at a sampling time.

usable for doing trial operations which need the fish be anesthetized.

Although Anesthetic induction as well as recovery phase was not significantly affected by concentration, anesthetic and recovery time was respectively, decreased and increased by elevating anesthetic agent dosage.

Longer recovery time which was observed in fish anesthetized with clove oil could be an additional advantage in activities such as morphological evaluations, biopsy and stripping which are required long handling periods outside the water (Rodrı´guez-Gutierrez and Esquivel-Herrera, 1995; Anderson *et al.*, 1997; Munday and Wilson, 1997; Prince and Powell, 2000; Sladky *et al.*, 2001)

Effects of water temperature were obvious in all of the concentrations. Water temperature significantly ($P<0.05$) affected on both anesthetic and recovery time. The higher the water temperature, the lower the anesthetic and recovery time. Since environmental factors affect the efficacy of anesthetics in fish, it is not surprising that the relationship between clove oil dosage and water temperature was also significant ($P<0.05$) regarding anesthetic and recovery time.

As a poikilothermic animal; body temperature of fish closely follows their environments which result in temperature-related physicochemical passage of the drug into the fish (Coyle *et al.*, 2004). Therefore, at lower water temperatures, higher doses or longer exposure times to anesthetic agents required due to the decrease in absorption rate (Ackerman *et al.*, 2005).

This suggests that the levels of clove oil used in our trial may have little or no effect on humans that consume fish treated with this anesthetic. What's more, the other major advantage of clove oil is its price and not unpleasant to work with.

Plasma Cortisol as well as glucose are physiological indicators of stress in fishes and their interactive effects on metabolism during recovery from stress have recently become a subject of more intense study (Cataldi *et al.*, 1998; Warren *et al.*, 2004; Barton *et al.*, 2005; Ribas *et al.*, 2007). In fact, the response to environmental stress is activation of the hypothalamic-pituitary-interrenal axis with an increase in the blood plasma of the steroid hormone, cortisol (Baker *et al*., 2005). We monitored Cortisol level at specific times before and after anesthetic exposure to elucidate how anesthetic solutions influence it. According to the efficacy of an ideal Anesthetic agent, it prevents increasing in cortisol level (Olsen *et al.*, 1995).

Although it is claimed that some anesthetics including clove oil blocks activation of the hypothalamo-pituitary-inter-renal (HPI) axis and releasing circulating cortisol in relation to the handling procedures (Iwama *et al.*, 1989; Ross and Ross, 1999; Ribas *et al.*, 2007), Elevating cortisol plasma upon replacement in recovery tank, as observed in our experiments, might be due to the low respiration after stageIII of anesthesia which result in respiratory acidosis as well as " hypoxia and consequently high blood cortisol level" As was demonstrated in fish anaesthetized with buffered TMS, 2-Phenoxyethanol, Benzocaine, Metomidate, and CO_2 (Iwama *et al.*, 1989; Molinero and Gonzalez,

1995). It suggests activation of HPI axis despite deeply anesthetized with clove oil (Pickering *et al.*, 1982; Thomas and Robertson, 1991). Clove oil was used to reduce stress before slaughter for comparing it's efficiently with hypothermia and asphyxia methods and similarity in plasma cortisol between unstressed and anesthetized group with this agent was observed (Ribas *et al*., 2007).clove oil might act much more quickly for inducing anesthesia in senegal sole, *Solea senegalensis*, than in persian sturgeon, *Acipenser persicus*, which result in depressing of cortisol response and its normal circulation. Clove oil did not block the cortisol response to stressors operations in sea bream, *Sparus aurata*, just like happening with other anaesthetics an similar to our trial (Tort *et al.*, 2002).

Hypoxia as a result of reduction in respiratory actions lead in physiological changes in the blood factors such as raising glucose and haematocrit (Hct) to combat with lowering in O_2 in circulation for breathing and survival (Tytler and Hawkins, 1981). Elevating plasma glucose as well as Hct and hemoglobin was similar to the reports for other anesthetized fish (Gomes *et al.*, 2001; Sladky *et al.*, 2001; Sandodden *et al.*, 2001; Ribas *et al.*, 2007).

White Blood Cell (WBC) was measured to evaluate clove oil effect on fish immune system. It showed a decline trend associated with arresting in anesthetic in stage III. We had already observed an increase in plasma cortisol concentration which is a Glucocorticoid hormone and can act as an immunosuppressive (Fast *et al.*, 2008), so it could suppress humoral factors and lead in declining circulating WBC along with elevating cortisol.

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