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Anaesthetic efficacy and physiological responses to clove oil-anaesthetized kelp grouper *Epinephelus bruneus*

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Anaesthetic efficacy and physiological responses to clove oil-anaesthetized kelp grouper *Epinephelus bruneus*

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Korean abstract

자바리, *Epinephelus bruneus*에 대한 clove oil의 18, 22 및 26℃ 수온 조건에서의 마취효과와 혈액생리학적(plasma cortisol, plasma glucose) 반응을 조사하였다.

3분 내외의 완전마취(Stage A7)와 10분 내외의 완전회복(Stage R6)을 기준으로, clove oil은 마취 수온 18℃ 조건 하에서는 250~300 ppm이, 마취 수온 22℃ 조건 하에서 150~200 ppm이, 그리고 마취 수온 26℃ 조건 하에서는 50~100 ppm이 자바리의 마취 적정 농도로 판명되었다. 자바리는 clove oil의 마취 농도가 높아질수록 마취시간은 짧지만 상대적으로 회복시간이 길어지는 경향이 있었고(P<0.05), 동일 농도의 clove oil에서는 마취 수온이 낮을수록 마취시간과 회복시간이 길어지는 경향을 나타내었다(P<0.05).

마취농도에 따른 plasma cortisol 농도는 마취 수온 22℃의 150 ppm clove oil에서 마취 12시간 후 4.24±1.571 μg/dL로 가장 높은 값을 보였고(P<0.05), plasma glucose 농도는 마취 2시간 후 92.7±9.61 mg/dL로 가장 높은 값을 보였다(P<0.05). 마취 72시간 후에는 실험 개시전 수준으로 회복되었다. 본 연구의 결과는 다방면에서 마취가 필요시되는 자바리 양식산업에 유용하리라 사료된다.

I. Experiment

Anaesthetic efficacy and physiological responses to clove oil-anaesthetized kelp grouper *Epinephelus bruneus*

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39. 877-884.

Aquaculture Research, 2008, **39**, 877–884 doi: 10.1111/j.1365-2109.2008.01941.x

Anaesthetic efficacy and physiological responses to clove oil-anaesthetized kelp grouper *Epinephelus bruneus*

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Abstract

The efficacy of clove oil as an anaesthetic and at producing a physiological response (plasma cortisol and glucose) was evaluated in the kelp grouper, *Epinephelus bruneus*. To acquire complete anaesthesia in less than 3 min and recovery in < 10 min, three doses of clove oil were tested at 18, 22 and 26 °C. Although higher anaesthetic doses resulted in shorter induction times and longer recovery times, and a lower temperature resulted in longer anaesthesia induction and slower recovery, we found the optimal dose and administering temperature of clove oil to be 250–300 mg L^{-1} at water temperature of 18 °C, 150–200 mg L^{-1} at water temperature of 22 °C and 50–100 mg L^{-1} at water temperature of 26 °C respectively. Following the administration of 150 mg L^{-1} of clove oil at 22 °C, the plasma cortisol level was highest (4.24 ± 1.571 μg dL^{-1}) after 12 h and the plasma glucose was highest (92.7 ± 9.61 mg dL^{-1}) after 2 h. These results should be useful to the aquaculture industry, where anaesthesia is necessary for a range of activities.

Keywords: anaesthesia, blood physiological response, clove oil, kelp grouper (*Epinephelus bruneus*), plasma cortisol, plasma glucose

Introduction

Farmed fish can be subjected to a range of stressors including handling, confinement, transport, medication, density of breeding, quality of water and changes in water temperature and salt levels (Singley & Chavin 1971; Fryer 1975; Donaldson 1981; Wedemeyer & Mcleay 1981). When stress is induced by these factors, fish react by consuming more energy, which subsequently drives a response of excess catecholamine and cortisol secretion, impacting considerably on the maintenance of homeostasis (Clarke, Shelboune & Brett 1981; Barton & Iwama 1991; Pickering 1992, 1993). Anaesthesia can decrease stress when fish are subjected to blood sampling, immobilization, handling, injection of vaccines and antibacterial substances, medical treatment for disease, artificial spawning, transport and sorting (Westerfield 1993).

In recent years, clove oil has been used widely in the aquaculture industry because it is safe, inexpensive, non-toxic in the environment and does not require a withdrawal period compared with other anaesthetic chemicals (Kang, Kim, Kim, Lim, Sim, Kim & Park 2005). However, there is no regulation for anaesthetic in Korea to date. Clove oil has been studied as an anaesthetic in a number of species including medaka, *Oryzias latipes* (Endo, Ogishima, Tanaka & Ohshima 1972), goldfish, *Carassius auratus* (Endo *et al.* 1972), carp, *Cyprinus carpio* (Hikasa, Takase, Ogasawara & Ogasawara 1986), rabbitfish, *Siganus lineatus* (Soto & Burhanuddin 1995), rainbow trout, *Oncorhynchus mykiss* (Keene, Noakes, Moccia & Soto 1998), channel catfish, *Ictalurus punctatus* (Waterstrat 1999), Atlantic salmon, *Salmo salar* (Chanseau, Galiay & Oules 2002) and sockeye salmon, *O. nerka* (Woody, Nelson & Ramstad 2002). The anaesthetic effect of clove oil was also confirmed in American lobster, *Homerus americus* (Waterstrat 2005), fresh prawn, *Macrobrachium resenbergii* (Coyle, Dasgupta, Tidweel, Beavers, Bright & Yasharian 2005) and common octopus, *Octopus minor* (Seol, Lee, Im & Park 2007). The active ingredient (70–80%) in clove

oil is eugenol [2-methoxy-4-2-(2-propenyl)-phenol] (British Pharmacopoeia 1993). It is generally used as an analgesic and disinfectant in dentistry (Curtis 1990) and as an additive in perfumes (Maura, Pino & Ricci 1989).

Kelp grouper, *Epinephelus bruneus* (Bloch) (order Perciformes, family Serranidae), is a sedentary marine fish that lives in rocky areas of shallow coastal regions and in some deeper areas, ranging to southern seas including Korea's Cheju Island, the southern part of Japan, China, the Philippines and India (Choi, Kim & Park 2002). The aquaculture of kelp grouper has been attempted in Korea, but it is difficult yet. Until now, no study has investigated the effects of anaesthesia and possible physiological stress on this species.

Therefore, the aim of this study was to determine the optimum concentration of anaesthetic clove oil for kelp grouper over a range of temperature and concentration conditions. The physiological responses of plasma cortisol and plasma glucose were also subsequently investigated.

Materials and methods

Experimental fish

In May 2006, kelp grouper (*E. bruneus*) were obtained from the Gyeongsangnam-do Fisheries Resources Research Institute, Republic of Korea. The fish were transported and reared in a recirculating culture system in the Fishery Genetics and Breeding Science Laboratory of the Korea Maritime University. The recirculating culture system consisted of five 1100-L circular tanks, one 1100-L filtering tank, an aeration system and a temperature control system. Culture water was partially replaced with sand-filtered, aerated seawater (salinity 34 ± 0.6 ppt, pH 7.6 ± 0.5, dissolved oxygen 8.5 ± 0.7 mg L^{-1}, ammonia 0.006 mg L^{-1}) every weekend.

The anaesthetic effect and blood physiological response experiment began in January 2007. Kelp grouper used in the experiment were measured using a digital vernier caliper (CD-20CP, Japan) and an electronic balance (JW-1, Republic of Korea). Average body length and weight were found to be 16.1 ± 1.48 cm and 105.3 ± 11.43 g respectively.

Investigation of the anaesthetic effect

The anaesthetic effect of clove oil was investigated at six concentrations, 50, 100, 150, 200, 250 and 300 mg L^{-1}, and three water temperatures 18, 22 and 26 °C. The stock solution of clove oil (Sigma, St Louis, MO, USA) was dissolved in 95% ethanol at a ratio of 1:10 (Cho & Heath 2000). Fish were fasted for 24 h before the start of the study and stocked in 12-L plastic tanks (quantity 10 L) in a static condition such as constant temperature using 500-L aquarium under temperature control system. Subsequently, 10 fish at a time were exposed to different combinations of water temperature and anaesthesia concentrations respectively. All experiments were performed in triplicated. The anaesthesia levels and recovery times of fish were measured in seconds using a stopwatch.

Anaesthetic effect criterion

The anaesthetic effect decision-based table (Table 1) was modified from data reported by Summerfelt & Smith (1990) and Woolsey, Holcomb and Ingermann (2004). Anaesthesia time was determined from the time when the fish were stocked in anaesthetized water to the time of the Stage A7 state, in which opercular movement ceased. Recovery time was determined from the time when the fish were stocked in recovery water to the time of the Stage R6 state, in which normal swimming and responsiveness to visual stimulation recommenced.

Table 1 Stage of anaesthesia induction and recovery in clove oil efficacy tests performed in kelp grouper, *Epinephelus bruneus* modified from Summerfelt and Smith (1990) and Woolsey *et al.* (2004)*

Stage	Characteristic behavior
Anesthesia	
A1	Normal swimming; opercular movement and normal general movement
A2	Swimming speed slowed; rolling from side to side
A3	Partial loss of equilibrium; swimming erratic
A4	Complete loss of equilibrium; swimming perfectly inside out; pectoral fin, pelvic fin and dorsal fin movement stop
A5	Little sedation; anal fin and tail fin movement stop
A6	Perfect sedation; only opercular movement
A7	Opercular movement ceased
Recovery	
R1	Resume opercular movement
R2	Preferential movement of pectoral fin and tail fin
R3	Dorsal fin, pelvic fin and anal fin movement
R4	Swimming perfectly inside out
R5	Swimming erratic; redress the balance
R6	Normal swimming; responsiveness to visual stimuli

*Body weight and standard length were 105.3 ± 11.43 g and 16.1 ± 1.48 cm, respectively, in the experiment.

Blood physiology response with the passage of time after anaesthesia

For this experiment, the food supply was disrupted 24 h ago before sampling. The blood physiological response was measured with the passage of time after the fish were anaesthetized with a clove oil concentration of 150 mg L^{-1} at a water temperature of 22 °C that is middle water temperature and middle concentration to receive representative value in result that have anaesthesia experiment by each water temperature and concentration. Blood samples were extracted from five fish at control, 0 (pre), 1, 2, 6, 12, 24, 48 and 72 h post anaesthesia respectively. All experiments were performed in triplicate. The fish used in this experiment were not involved in the anaesthetic effect experiment.

Blood sampling and analysis

Blood was collected from the caudal vasculature using a disposable syringe (3 mL, Sung Shim Medical, Bucheon, Republic of Korea) with heparin sodium (Shin Poong Pharm, Ansan, Republic of Korea) within 1 min. Blood was kept for 10 min at normal temperature before centrifugation (Centrifuge Micro 17R, Hanil Science Industrial, Incheon, Republic of Korea) for 10 min at 22250 *g*. The collected plasma was transferred to another 1.5 mL microtube and kept at −70 °C in a super low temperature refrigerator (CLN-50UW Nihon Freezer, Nihon, Japan) before analysis.

The plasma cortisol concentration was measured using a 1470 WIZARD Automatic Gamma Counter (Cobra Packard, Ramsey, MN, USA) after the antigen antibody response was derived using a Coat-A-count TKCO Cortisol RIA Kit (DPC, Los Angeles, CA, USA) according to the Donaldson (1981) method. The plasma glucose concentration was analyzed, according to Raabo and Terkildsen (1960; Kit 510, Sigma, St Louis, MO, USA), through evaluating the production of H_2O_2 by glucose oxidase in the presence of *o*-dianisidine as an absorbance increase at 450 nm.

Statistical analysis

One- and two-way analysis of variance (ANOVA) were used to test for the significance ($P < 0.05$) of the effects of temperature and clove oil concentration. The differences among groups were analyzed by ANOVA using the SPSS statistics package (SPSS 9.0, SPSS, Chicago, IL, USA), and multiple comparisons were performed using Duncan's multiple range test (Duncan 1955).

Results

Anaesthetic effect

Table 2 shows the parameters associated with the effects of clove oil as an anaesthetic at each concentration and water temperature. The anaesthetic time was significantly ($P < 0.05$) affected by temperature and clove oil concentration, and decreased

Table 2 Effects of clove oil dose and starting water temperature on anesthesia among kelp grouper *Epinephelus bruneus*

	Anesthetic time (s)*			Recovery time (s)*		
Dose (mg L^{-1})	18 °C	22 °C	26 °C	18 °C	22 °C	26 °C
050	91.2 ± 8.36^{a}	63.4 ± 4.55^{a}	48.4 ± 3.41^{a}	31.3 ± 3.13^{f}	18.7 ± 0.82^{f}	14.0 ± 0.82^{f}
100	81.2 ± 5.79^{b}	59.0 ± 3.46^{b}	41.4 ± 3.53^{b}	35.6 ± 2.01^{e}	26.0 ± 1.41^{e}	15.0 ± 0.82^{e}
150	72.6 ± 4.86^{c}	53.7 ± 3.40^{c}	40.6 ± 3.24^{c}	48.8 ± 3.01^{d}	27.4 ± 1.35^{d}	18.0 ± 0.67^{d}
200	69.3 ± 5.54^{d}	45.0 ± 2.58^{d}	40.0 ± 2.87^{d}	50.2 ± 3.43^{c}	35.3 ± 2.11^{c}	22.0 ± 1.49^{c}
250	55.7 ± 3.20^{e}	39.9 ± 2.56^{e}	35.2 ± 2.15^{e}	63.2 ± 3.99^{b}	40.0 ± 2.87^{b}	23.0 ± 1.41^{b}
300	50.2 ± 3.01^{f}	39.0 ± 2.49^{f}	29.8 ± 2.30^{f}	67.5 ± 4.09^{a}	42.8 ± 3.01^{a}	26.0 ± 1.15^{a}

	Two-way ANOVA									
	DF	ANOVA SS	Mean square	*F*-value	*P*-value	DF	ANOVA SS	Mean square	*F*-value	*P*-value
Temperature	02	30 034.5	15 017.2	944.52	<0.0001	02	29 111.2	14 555.6	2598.07	< 0.0001
Dose	05	15 868.8	3173.8	199.61	<0.0001	05	14 850.2	2970.0	530.13	< 0.0001
Interaction	10	2308.5	230.8	14.52	<0.0001	10	3746.8	374.7	66.88	< 0.0001

*Each value is mean ± standard deviation ($n = 10$). Values in the same column not sharing common superscripts are significantly different ($P < 0.05$).

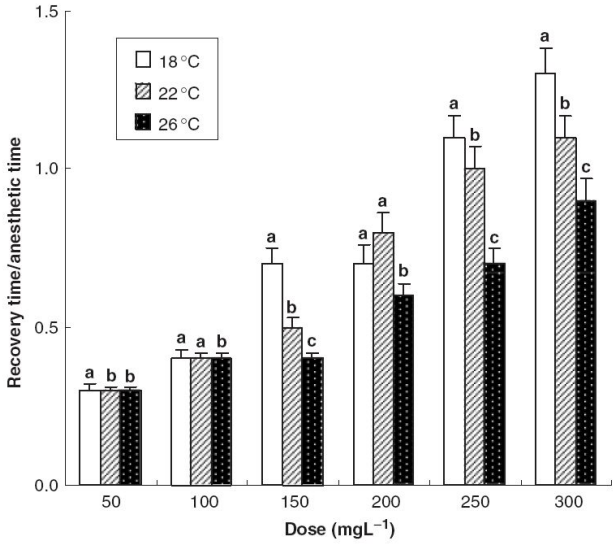

Figure 1 Effect of clove oil dose and water temperature on recovery time/anaesthetic time ratio among kelp grouper, *Epinephelus bruneus*. Different letters on the bars are significantly different ($P < 0.05$).

linearly as the concentration and temperature increased. We also found significant relationships ($P < 0.05$) between water temperature and concentration and anaesthetic time. At each water temperature, as the concentration of clove oil increased, the anaesthetic time decreased. Furthermore, as the water temperature increased, the anaesthetic time also decreased. The recovery time was significantly affected ($P < 0.05$) by water temperature and clove oil concentration of clove oil. The interaction of water temperature and dose on recovery time was also significant ($P < 0.05$). As the concentration of the anaesthetic increased, recovery time significantly increased ($P < 0.05$). However, as the water temperature increased, the recovery time significantly decreased ($P < 0.05$).

The anaesthesia concentrations of clove oil and results for the ratio of recovery time in relation to the anaesthesia time according to the anaesthesia water temperature are shown in Fig. 1. In terms of the trends of the recovery time and anaesthesia time according to the anaesthesia water temperature, few differences are seen in the ratio of recovery time to anaesthesia time at different water temperatures at anaesthesia concentrations of 50 and 100 mg L^{-1}. However, when anaesthesia concentrations of 150, 200, 250 and 300 mg L^{-1} were used, the anaesthesia time increased more than the recovery time as the water temperature rose ($P < 0.05$).

Blood physiology response with the passage of time after anaesthesia

The plasma cortisol and plasma glucose concentrations with the passage of time after anaesthesia are shown in Fig. 2.

Plasma cortisol

The mean plasma cortisol concentration was 0.28 ± 0.057 μ dL^{-1} before the initiation of the experiment. When the fish were moved to the recovery water tank after anaesthetization with clove oil at 150 mg L^{-1} and water temperature at 22 °C, the plasma cortisol concentration was 1.39 ± 0.262 μ dL^{-1} to pre (0 h), 0.51 ± 0.205 μ dL^{-1} to 1 h, 0.54 ± 0.321 μ dL^{-1} to 2 h and 0.69 ± 0.777 μ dL^{-1} to 6 h; these differences were not statistically significant ($P > 0.05$). However, the plasma cortisol concentration increased by 4.24 ± 1.571 μ dL^{-1} in 12 h, and decreased by 3.51 ± 0.832 and 2.82 ± 0.834 μ dL^{-1} in 24 and 48 h, respectively, although a high plasma cortisol concentration was still present ($P < 0.05$). The plasma cortisol concentration in 72 h was found to be 0.44 ± 0.426 μ dL^{-1}, and was similar to that of the control group ($P > 0.05$).

Plasma glucose

The plasma glucose concentration of fish was 31.2 ± 2.52 mg dL^{-1} before the initiation of the

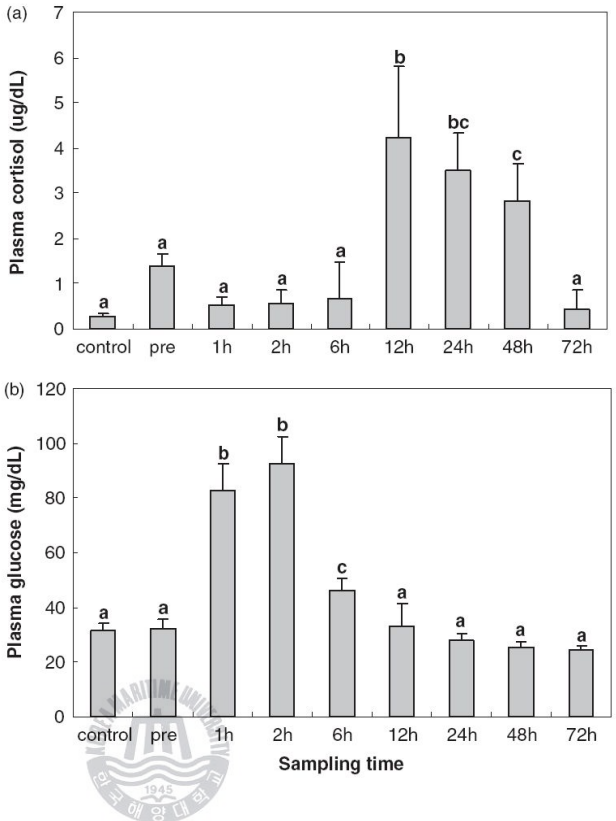

Figure 2 Post-recovery physiological measurements (means ± SD) of kelp grouper, *Epinephelus bruneus*: (a) plasma cortisol; (b) plasma glucose. Different letters on the bars are significant difference between treatment and control groups at a sampling time.

experiment. When fish were moved to the recovery water tank after anaesthetic administration, the mean plasma glucose concentration was shown to be 32.0 ± 3.61 mg dL^{-1} at pre (0 h), similar to that of the control group ($P > 0.05$).

The plasma glucose concentration increased by 83.2 ± 9.64 mg dL^{-1} in 1 h, and increased by 92.7 ± 9.61 mg dL^{-1} in 2 h ($P < 0.05$). The plasma glucose concentration after 6 h showed a decrease of 46.6 ± 4.58 mg dL^{-1}, but was still higher than that of the control group ($P < 0.05$). The plasma glucose concentrations at 12, 24, 48 and 72 h were 33.3 ± 8.08, 27.7 ± 2.89, 25.3 ± 2.08 and 24.3 ± 1.53 mg dL^{-1}. These values were not significantly different from those of the control group ($P > 0.05$).

Discussion

Anaesthesia is used to tranquilize fish for weight and length measurement, label and tag injection, physiology and ethology study, wound healing, gathering, photography, artificial spawning, injection of vaccine and antibiotic and live fish transport, all of which contribute to the fields of biological research, aquacultural research and minimization of stress in the fish industry (Summerfelt & Smith 1990; Park, Kim, Kim & Kim 1988; Park, Lim & Choi 1998a; Park, Kim, Jung & Im 1998b; Park, JO, Lee, Kim, Park, Hur, YOO & Song 2003; Park, Hur, Song, Im & Johnson 2004; Hur, Park, Kho & Chang 2005). Treatment with excessive anaesthesia is very stressful to fish, causing abnormal metabolic rate, oxygen consumption, blood pressure and blood physiological responses. Moreover, these side-effects can last for hours after fish recover from anaesthesia (Summerfelt & Smith 1990). Optimum anaesthetic concentrations can minimize the negative impact and thus reduce stress in fish.

In result of Fig. 1, if the ratio of recovery time to anaesthesia time is higher than 1, the recovery time is longer than the anaesthesia time; if the ratio is 1,

then the recovery time and the anaesthesia time are the same. If the recovery time is shorter than the anaesthesia time, the ratio is < 1. Because the ratio of recovery time to anaesthesia time increases as the anaesthesia concentration is increased, the anaesthesia time is shortened as the anaesthesia concentration increases, but the recovery time increases relatively. In contrast, the anaesthesia time increased as the anaesthesia concentration was reduced, but the recovery time was relatively shortened ($P < 0.05$).

Optimum anaesthetic concentrations are usually expected to induce anaesthesia within 3 min and recover within 10 min (Gilderhus & Marking 1987; Son, Park, Myeong, Kim, Kim, Jo & Jeon 2001; Park *et al.* 2003). When considering the water temperature, all anaesthesia experiment results satisfy the above condition. So, we decided optimum anaesthesia concentration that is shown as anaesthesia time within 1 min and fast recovery by water temperature. The optimal anaesthesia concentrations for kelp grouper were 250–300 mg L^{-1} at 18 °C, 150–200 mg L^{-1} at 22 °C and 50–100 mg L^{-1} at 26 °C respectively.

In this study, kelp grouper was shown to be sensitive to the anaesthetic effect of clove oil, for both concentration and temperature. Similar results were reported for goldfish, *Carassius auratus*, muddy loach, *Misgurnus anguillicaudatus*, Chinese minnow, *Rhynchocypris oxycephalus*, amur minnow, *Rhynchocypris steindachneri* and pacific cod, *Gadus macrocephalus* (Mattson & Riple 1989).

Plasma cortisol and plasma glucose are recognized as useful indicators of stress in fish (Schreck 1982). Clove oil is not, but among study that use other fish anaesthetic, notable increases in plasma cortisol and plasma glucose levels were reported in red drum, *Sciaenops ocellatus* simultaneously exposed to MS-222 and Quinaldine anaesthetic (Massee, Rust, Hardy & Stickney 1995).

In Fig. 2, the plasma cortisol concentration of anaesthetized kelp grouper did not return to normal until 48 h. This agrees with the result that the effect of cortisol caused by sudden stress continued for 48 h, even if brown trout, *Salmo trutta*, rainbow trout, *Salmo gairdneri* were exposed to stress for a relatively short period of time (Pickering, Pottinger & Carragher 1989).

Barton & Iwama (1991) stated that 'Usually, phenomenon that plasma cortisol concentration of fishes rises by stress is first order reaction, phenomenon that plasma glucose concentration rises is result of second-order first order reaction by hormone rise reaction by stress'. This trend has been reported in the grey mullet, *Mugil cephalus* (Chang & Hur 1999).

In this study, the plasma glucose concentration showed an increase earlier than the plasma cortisol concentration, unlike that reported by Chang & Hur (1999). The plasma cortisol concentration later increased while the plasma glucose concentration decreased. Such different results seem to be caused by different kinds of species and stresses imposed on fish.

In our study, after exposure to an anaesthesia concentration of 150 mg L^{-1} at 22 °C, it took 3 days for the plasma cortisol and plasma glucose concentrations to return to the levels seen before exposure. The anaesthetic effect of clove oil in fish can last for several hours after relocating fish to recovery tanks in this study.

In general, the main considerations in the anaesthetization of fish are safety, efficiency, price, toxicity, physiological response, duration of withdrawal and restriction of use (Bell 1987; Park *et al.* 2003; Kang *et al.* 2005). The main ingredient of clove oil, eugenol, can be regarded as highly safe for human use. Because it is 'generally recognized as safe (GRAS),' it is used as a food additive and an analgesic and disinfectant in dentistry (Schnick, Meyer & Walsh 1986).

Future investigations should focus on the comparative physiological reactions in kelp grouper, induced by clove oil and other fish anaesthetics.

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