Olfactory nerve response of masu salmon and rainbow trout to clove oil and MS-222

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Running headline: Olfactory response of salmon to anaesthetics

Key words: clove oil, MS-222, anaesthetic, salmon, olfactory nerve response.

Abstract

Two anaesthetics, clove oil and MS-222, were examined for their effects on the olfactory nerve response of masu salmon (*Oncorhynchus masou*) and rainbow trout (*O. mykiss*). Exposing fish to clove oil for 3 minutes at concentrations of 50 mg/L and 100 mg/L, or for 10 minutes at 50 mg/L, did not significantly reduce their olfactory response. Directly applying clove oil anaesthesia to the olfactory epithelium significantly reduced olfactory response, though olfactory response recovered to 70% and 52% of pre-treatment levels in masu salmon and rainbow trout, respectively, after 20 minutes. After a three minute exposure to unbuffered MS-222 (100 mg/ml), olfactory responsiveness continued to decline over time but no such long-term effects were observed after exposure to clove oil or buffered MS-222. Clove oil appears to be an effective and safe anaesthetic for salmonids with no long-term impact on their olfactory response, which plays a crucial role in their life history.

INTRODUCTION

Fisheries research often requires handling research subjects, potentially reducing their immunological capacity (Ellis, 1981; Fries, 1986; Schreck *et al*., 1989; Mommsen *et al*., 1999). Anaesthetics are routinely used to block the

hypothalamus-pituitary–interrenal axis and to prevent fish from reacting to additional stressors (Olsen *et al*., 1995). It is generally assumed that anaesthetics do not cause long term changes in fish behavior and that normal sensory interpretation, including olfaction, rapidly resumes after recovery. Only one study has shown long term reduction in olfaction from a widely used fish anaesthetic: tricaine methane sulfonate (MS-222) which reduced olfaction in channel catfish (*Ictalurus punctatus*) for up to 28 days (Lewis *et al*., 1985). Subsequent assays showed no influence of MS-222 on the olfaction-mediated behaviors of homing in chinook salmon (*Oncorhynchus tshawytscha)* or avoidance to L-serine in coho salmon (*O. kisutch)* (Quinn *et al*., 1988). MS-222 is currently the only highly effective anaesthetic approved for use on food fishes by the U.S. Food and Drug Administration (FDA). However, MS-222 is expensive and requires a 21 day depuration period prior to consumption or release of fish, limiting its applicability and motivating fisheries researchers to examine cheaper alternatives with a zero withdrawal time.

Clove oil is a promising anaesthetic for use on teleosts. Clove oil is an extract of the *Eugenia aromatica* tree whose active ingredient is eugenol (4-allyl-2-methoxyphenol); it is commercially available and inexpensive. Efficacy studies thus far suggest that clove oil and its derivatives (e.g. Aqui-S ^{TM}, Ross and Ross,

1999; eugenol, and isoeugenol) are effective fish anaesthetics (Soto & Burhanuddin, 1995; Taylor & Roberts, 1999; Woody *et al*., 2002; Iversen *et al*., 2003). Although it appears to be an ideal anaesthetic (see criteria by Marking & Meyer, 1985), few studies have examined its effects on fish physiology. The limited studies have indicated both positive (Anderson *et al*., 1997; Pirhonen & Schreck, 2003; Wagner *et al*., 2003) and negative results (Davidson *et al*., 2000). Woody *et al*. (2002) raised concern regarding potential negative impacts to salmon olfaction and therefore homing ability. To date, no studies have been conducted on this important question.

Clove oil's effect on olfactory nerve response was examined in masu salmon (*O. masou*) and rainbow trout (*O. mykiss*), with specific focus on both time course changes and recovery changes after treatment. Olfactory nerve response differences were also compared for masu salmon across treatments of clove oil, MS-222, and MS-222+ $NaHCO_3$.

MATERIALS AND METHODS

Experimental animals

One-year-old masu salmon of the Mori strain and 1-year-old rainbow trout of the Date strain were used in the experiments. All fish were hatched and reared at the Toya

Lake Station, Hokkaido University, Hokkaido, Japan. Masu salmon fork lengths averaged 21.42 cm (SE = 2.95 cm) and weights averaged 123.60 g (SE = 23.44 g). Rainbow trout fork lengths averaged 23.8 (SE = 4.49 cm) and weights averaged 169.40 g (SE = 5.90 g). All experiments were repeated using 3-5 fish per experiment, each fish was used for a single trial.

Olfactory Stimulants

Fish were tested at water temperatures between 8 - 11^{o}C. All pond water (PW) originated from natural spring water used in the study. Water chemistry of PW was reported; 40mg・L^{-1} total hardness as $CaCO_3$, pH 6.1, conductivity 0.14 $mScm^{-1}$ turbidity 0.0 nephelometric turbidity units (NTU), dissolved oxygen 9.1mgL^{-1}. The compositions of amino acid and related substances in the pond water were as follows (nM): Phosphoserine, 8.92; taurine, 5.66; L-aspartic acid, 2.72; L-threonine, 5.15; L-serine, 2.27; L-glutamic acid, 2.07; glycine, 4.97; L-alanine, 3.02; L-valine, 1.05; L-isoleucine, 7.48; L-lysine, 1.15; Phosphoethanolamine, 1.73; L-tyrosine 9.72; L-phenylalanine 5.36; β-alanine 6.08; γ-amino butyric acid, 1.45; Etanolamine, 3.4; 1-methyl-L-histidine, 4.2; L-histidine, 9; L-anserine, 1.9; L-glutmine 1.07.

Mixtures of L-Alanine (L-Ala) in PW were used as the stimulus to assess olfactory nerve response before (control) and after anaesthetic exposure. L-Ala concentration

varied with the experiment. Clove oil was purchased from Wako Pure Chemical Industries (Tokyo, Japan). And L-Ala and MS-222 were purchased from Wako Pure Chemical Industries (Tokyo, Japan), Sigma Chemical Co. (St Louis, USA).

Anaesthetics

Clove oil and MS-222 test solutions were prepared with PW and stirred 30 min to 1 hour. New solutions were prepared every other day and stored in a refrigerator between uses. Anaesthetic concentration varied with the experiment.

Experimental Protocol

Integrated olfactory nerve response was measured using the electrophysiological techniques of Sveinsson & Hara (1990). Fish were immobilized with an intramuscular injection of gallamine triethiodide (Sigma, St. Louis, MO, USA; 3 mg/kg body weight). Local anaesthesia (lidocaine) was applied where fish were affixed to the study chamber and at the surgery site. Gills were bathed through the mouth with an aerated solution of tricaine methane sulfonate (MS-222; 70 mg/L) which was not allowed to contact olfactory rosettes. A stereotaxic chamber kept exposed portions of the fish moist throughout the experiment.

A portion of the skull was surgically removed to expose the proximal olfactory nerve and bulbs. Twin tungsten electrodes were inserted into the olfactory nerve to

record response to olfactory stimulus. A grand electrode filled with 3M KCl agar (2%) bridged to an Ag-AgCl electrode was placed on the dorsal skin. Electrodes remained in place for the duration of the experiment. After electrode placement, the olfactory rosettes were rinsed for 30 min with PW.

Recording olfactory nerve response

Olfactory nerve response was determined by monitoring electrical nerve response to different chemical concentrations and exposure durations. Irrigating and stimulating solutions were applied to the olfactory epithelium via a stainless steel tube. The nerve response signal was amplified by an AC preamplifier (MOD. DAM-5A, W-P Instruments, Sarasota, FL, USA) at 300-3 KHz and integrated by an electric integrator (time constant = 0.3 seconds). Integrated olfactory nerve responses were recorded by a pen recorder.

A fish's olfactory response magnitude was defined as the height of the tallest spontaneous peak in the integrated nerve response. Olfactory response to the stimulus was measured prior to anaesthetic exposure and taken as the control response magnitude. The stimulus was re-applied and olfactory response remeasured at various times after exposure to the anaesthetic, as determined by each experiment. The magnitudes of olfactory response to the stimulus was standardized to the control response.

Pilot Study

A pilot study was conducted to determine effective clove oil anaesthetic exposure times and recovery periods in both masu salmon and rainbow trout. Fish had reached a state where it did not react to handling define as anesthesia and showed ability to remain upright, normal swimming behavior as recovery.

Fish were placed in a plastic tub containing 20 L of fresh water and 50 mg/L of clove oil, and time to anaesthesia was measured. The fish was then transferred to an aerated fresh water tank, and time to recovery was measured.

Average anaesthetization time was about 3 minutes, so minimum anaesthesia treatment exposure time was set at 3 minutes. Average recovery time was about 6 minutes, so each experiment recorded olfactory nerve response for at least 6 minutes after anaesthesia treatment exposure.

Experiments

Four experiments were conducted. Experiment 1 assessed the smallest stimulus concentration in which a change in olfactory nerve response was detected after exposure to anaesthetic. The experiment exposed rainbow trout to different concentrations of L-Ala (10^{-3}-10^{-7} M) before and after exposure to 100 mg/L clove oil treatment for 3 minutes.

Experiment 2 compared the olfactory nerve response to the stimulus (10^{-4} M L-Ala) before and after exposure to three clove oil treatments: 50 mg/L exposure for 3 minutes, 50 mg/L exposure for 10 minutes, 100 mg/L exposure for 3 minutes. Response was measured at 0, 2, 4, 6, and 8 minutes. The experiment was conducted with both masu salmon and rainbow trout.

Experiment 3 compared the olfactory nerve response to the stimulus (10^{-4} M L-Ala) before, in the presence of 50 mg/L clove oil, and 20 minutes after the anaesthetic treatment. The experiment was conducted with both masu salmon and rainbow trout.

Experiment 4 compared the olfactory nerve response to the stimulus (10^{-4} M L-Ala) before and after exposure to three different anaesthetic treatments: 50 mg/L clove oil, 100 mg/L MS-222, and 100 mg/L MS-222 added to 100 mg/L $NaHCO_3$ (MS-222+ $NaHCO_3$), each using a 3 minute exposure. Response was measured at 0, 10, 20, 30, 40, and 50 minutes after treatment. The experiment was conducted on masu salmon.

Data Analyses

Standardized response magnitudes were averaged across all fish in an experiment and means ± SE are reported. One-way ANOVA was used to assess changes in mean standardized response magnitudes during each experiment with a significance level of α

= 0.05. Typical integrated olfactory nerve response signals are displayed for most experiments.

Results

Experiment 1 did not reveal any significant differences in mean olfactory response magnitudes due to clove oil treatment (100 mg/L, 3 minute exposure), at any of the stimulus concentrations (Figures 1a, 1b). Though non-significant, a slight change in response was observed using the stimulus concentration 10^{-3}M L-Ala (Fig. 1b).

Experiment 2 did not reveal any significant changes through time in the mean olfactory response magnitudes of either species after exposure to any of the three clove oil treatments (50 mg/L for 3 minutes, 50 mg/L for 10 minutes, 100 mg/L for 3 minutes) (Figures 2a, 2b).

Experiment 3 revealed a significant decline in olfactory response in both species in the presence of 50 mg/L of clove oil followed by recovery toward control levels after 20 minutes (Figures 3a, 3b). Average response values recovered to 70% ($\pm$0.31) in masu salmon and 52% ($\pm$0.17) in rainbow trout 20 minutes after the clove oil treatment.

Experiment 4 revealed significant declines through time in mean olfactory response magnitudes in masu salmon after exposure to 100 mg/L MS-222 but not after

exposure to 50 mg/L clove oil or 100 mg/L MS-222 and 100mg/L $NaHCO_3$ (MS-222+ $NaHCO_3$) (Figures 4a, 4b).

DISCUSSION

While many studies have assessed the effect of anaesthetics, including clove oil and MS-222, on swimming performance and handling stress minimization, little is known about their impacts on salmonid olfaction (Ellis, 1981; Taylor & Roberts, 1999; Woody *et al*., 2002; Wagner *et al*., 2003). This study shows that clove oil anesthesia appears an effective anaesthetic for salmonids, with little short-term influence on olfactory nerve response.

For anadromous salmonids, olfaction is a critical sense for perception in the avoidance of predators (Rehnberg and Schreck, 1987; Brown and Smith, 1997), the recognition of conspecifics (Quinn and Busack, 1985; Griffiths and Armstrong, 2000), imprinting and homing to the natal stream (Hasler and Scholz, 1983; Dittman and Quinn, 1996). Chemicals that elicit the response from the olfactory organs of salmon include amino acids, steroids, bile acids, and prostaglandins (Hara, 1992). In general, amino acids are potent odorants for fish. The salmon olfactory organ responds to various species of amino acids, for example, rainbow trout respond to 10^{-8} M L-serine

and 10^{-7} M L-Ala (Caprio, 1982, 1988; Hara, 1982, 1992). Commonly occurring amino acids, such as glycine, alanine, and serine, act as feeding stimulants in wide range of fish species (Sorensen and Caprio, 1998). Additionally, L-Ala was one of the highly stimulatory amino acids for rainbow trout (Hara, 1975). Thus L-Ala is major odors for salmonids, we used L-Ala as stimulus.

In masu salmon and rainbow trout it had no significant impact on olfactory sensitivity to stimuli (Figure 1b), nor any significant reduction on olfactory nerve responses (Figure 2b). This is an important trait for a potential anaesthetic given the crucial role olfaction plays in salmon survival, migration, and reproductive behavior (e.g. Wisby & Hasler, 1954).

When stimulating solutions were applied to the olfactory epithelium during direct anaesthesia by clove oil, olfactory nerve responses declined significantly. But after the clove oil treatment was discontinued, the response recovered to 70% and 52% before treatment in masu salmon and rainbow trout, respectively.

While olfactory nerve response recovered within an hour after treatment with clove oil or MS-222+$NaHCO_3$, it gradually and significantly declined after MS-222 treatment (Figure 4 b). MS-222 test solutions are acidic. Although lowering pH of solutions suppress the olfactory response (Hara, 1976), this may be due to destruction of cilia on

the olfactory sensory epithelia by exposure to MS-222, as occurred with channel catfish in an earlier study (Lewis *et al*., 1985), because the olfactory rosettes were rinsed with PW after anesthesia treatment.

The present study clearly demonstrates the safety of clove oil as an anaesthetic relative to olfaction in masu salmon and rainbow trout and is the first to report on the impacts of clove oil on olfactory nerve response of salmon. Given that clove oil appears more effective than MS-222 at reducing short-term handling stress (Wagner *et al*., 2003), in conjunction with the current results of no significant impact on olfaction suggests that clove oil is a safer anaesthetic in terms of impact on salmon olfactory nerve response. Thus clove oil and its derivatives may be considered an effective and safe anaesthetic for use on fishes in which olfactory function plays an important role in their life history, like salmonids. However, the study was undertaken using only L-Ala as the stimulus. Morphologically, there are three types of odrant receptor neurons (ORNs): ciliated, microvillar and crypt. For salmonids, ciliated ORNs are generalists that respond to all three odorant classes (pheromone, amino acid and bile salt) whereas microvillar ORNs are specialists that respond to amino acids (Sato and Suzuki, 2001). It is unknown what odorants correspond to crypt cells (Hansen and Zielinski, 2005), although sex pheromones have been hypothesized (Hamdani and Døving, 2006). Thus

clove oil may have an affect on the olfactory response to other type of odorants, and therefore further studies requires to ensure that clove oil doesn't reduce other type of olfactory stimuli.

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References

References

Anderson W.G., McKinley R.S. & Colavecchia M. (1997) The use of clove oil as an anaesthetic for rainbow trout and its effects on swimming performance. *North American Journal of Fisheries Management* **17**, 301-307.

Caprio J. (1982) High sensitivity and specificity of olfactory and gustatory receptors of catfishes to amino acids. In: *Chemoreception in Fishes* (ed. by T.J. Hara),

pp.109-134. Elsevier, Amsterdam.

Caprio J. (1988) Peripheral filters and chemoreceptor cells in fishes. In: *Sensory Biology of Aquatic Animals* (ed. by J. Atema, R.R. Fay, A.N. Popper, & W.N. Tavolga), pp. 339-363. Springer-Verlag, New York.

Davidson G..W., Davie P.S., Young G. & Fowler R.T. (2000) Physiological responses of rainbow trout *Oncorhynchus mykiss* to crowding and anaesthesia with AQUI-S™. *Journal of the World Aquaculture Society* **31**,105-114.

Ellis A.E. (1981) Stress and modulation of defense mechanisms in fish. In: *Stress and fish* (ed. by A.D. Pickering), pp 147-169. Academic Press, New York.

Fries C.R. (1986) Effects of environmental stressors and immuno-suppressants on immunity of *Fundulus heteroclitus*. *American Zoologist* **26**, 271-282.

Hamdani E.H. & Døving K.B. (2006) Specific projection of the sensory crypt cells I n the olfactory system in crucian carp, *Carassius carassius*. *Chemical Senses* **31**, 63–67.

Hansen A. & Zielinski B. (2005) Diversity in the olfactory epithelium of bony fishes: development, lamellar arrangement, sensory neuron cell types and transduction components. *Journal of Neurocytology* **34**, 183-208.

Hara T.J. (1975) Olfaction in fish. In: *Progress in Neurobiology* **Vol. 5. Part 4**. (ed. by

G.A. Kerrut & J.W. Phyllis), pp. 271-335. Pergamon Press, Oxford.

Hara T.J. (1976) Effects of pH on the olfactory responses to amino acids in rainbow rout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology* **54**A, 37-39.

Hara T.J. (1982) Structure-activity relationships of amino acids as olfactory stimuli. In: *Chemoreception in Fishes* (ed. by T.J. Hara), pp. 135-157. Elsevier, Amsterdam.

Hara T.J. (1992) Mechanism of olfaction. In: *Fish Chemo-reception* (ed. by T.J. Hara), pp. 150-170. Chapman Hall, London.

Iversen M., Finstad B., McKinley R.S. & Eliassen R.A. (2003) The efficacy of metomidate, clove oil, AQUI-S, and Benzoak ® as anaesthetics in Atlantic salmon (*Salmo salar* L.) smolts, and their potential stress-reducing capacity. *Aquaculture* **221**, 549-566.

Lewis D.H., Tarpley R.J., Marks J.E. & Sis R.F. (1985) Drug induced structural changes in olfactory organ of channel catfish *Ictalurus punctatus* Rafinesque. *Journal of Fish Biology* **26**, 355-358.

Marking L.L. & Mayer F.B. (1985) Are better anaesthetics need in fisheries? *Fisheries* **10**, 2-5.

Mommsen T.P., Vijayan M.M. & Moon T.W. (1999) Cortizol in teleost; dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and*

Fisheries **9**, 211-268.

Olsen Y.A., Einarsdottir I.E. & Nilssen K.J. (1995) Metomidate anaesthesia in Atlantic salmon, *Salmon salar*, prevents plasma cortisol increase during stress. *Aquaculture* **134**, 155-168.

Pirhonen J. & Schreck C.B. (2003) Effects of anesthesia with MS-222, clove oil and CO_2 on feed intake and plasma cortisol in steelhead trout (*Oncorhynchus mykiss*). *Aquaculture* **220**, 507-514.

Quinn T.P., Olson, A.F. & Konecki J.T. (1988) Effects of anaesthesia on the chemosensory behaviour of Pacific salmon. *Journal of Fish Biology* **33**, 637-641.

Ross L.G. & Ross B. (1999) *Synthetic and sedative techniques for aquatic animals 2nd edn* Blackwell, London.

Sato K. & Suzuki N. (2001) Whole-cell response characteristics of ciliated and microvillous olfactory receptor neurons to amino acids, pheromone candidates and urine in rainbow trout. *Chemical Senses* **26**, 1145-1156.

Schreck C.B., Solazzi M.F., Johnson S.L. & Nickelson T.E. (1989) Transportation stress affects performance of coho salmon, *Oncorhynchus kisutch*. *Aquaculture* **82**, 15-20.

Sorensen P.W. & Caprio J. (1998) Chemoreception. In: *The Physiology of Fishes 2nd edn* (ed. by Evans D.H.), pp. 375-405. CRC, Press LLC, New York.

Soto C.G.. & Burhanuddin (1995) Clove oil as a fish anaesthetic for measuring length and weight of rabbitfish (*Siganus lineatus*). *Aquaculture* 1**36**,149-152.

Sveinsson T. & Hara T.J. (1990) Analysis of olfactory responses to amino acids in arctic char (*Salvelinus alpinus*) using linear muitiple-receptor model. *Comparative Biochemistry and Physiology* **97A**, 279-287.

Taylor P.W. & Roberts S.D. (1999) Clove oil: an alternative anaesthetic for aquaculture. *North American Journal of Fisheries Management* **61**,150-155.

Wagner G.N., Singer T.D. & McKinley R.S. (2003) The ability of clove oil and MS-222 to minimize handling stress in rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquaculture Research* **34**, 1139-1146.

Woody C.A., Nelson J. & Ramstad K. (2002) Clove oil as an anaesthetic for adult sockeye salmon: field trials. *Journal of Fish Biology* **60**, 340-347.

Figure legends

Fig. 1a. Typical integrated olfactory nerve response of rainbow trout to different concentration of L-Alanine before and after clove oil treatment (100 mg/L, 3 minute exposure).

Fig. 1b. Relative magnitude of integrated olfactory nerve response of rainbow trout to different concentrations of L-Alanine before and after clove oil treatment. The values are means ± SE of data obtained from 4-5 fish.

Fig. 2a. Time course changes in typical integrated olfactory nerve response of masu salmon after exposure to three clove oil treatments.

Fig. 2b. Time course changes in relative magnitude of integrated olfactory nerve response of masu salmon and rainbow trout to different concentrations and anaesthetization times of clove oil. A: 50 mg/L, 3 minutes; B: 100 mg/L, 3 minutes; C: 50 mg/L, 10 minutes. The values are means ± SE of data obtained from 4-5 fish.

Fig. 3a. Typical integrated olfactory nerve response of masu salmon before, in the presence of 50 mg/L clove oil, and after 20 minutes of the clove oil treatment.

Fig. 3b. Relative magnitude of integrated olfactory nerve response of masu salmon and rainbow trout to 10^{-4}M L-Alanine before, in the presence of 50 mg/L clove oil,

and after 20 min of the clove oil treatment. The values are means ± SE of data obtained from 4 fish each.

Fig. 4a. Time course changes in typical integrated olfactory nerve response of masu salmon in different 3 minute anesthesia treatments: (A) 50 mg/L clove oil; (B) 100 mg/L MS-222; and (C) 100 mg/L MS-222 + 100mg/L $NaHCO_3$.

Fig. 4b. Time course changes in relative magnitude of integrated olfactory nerve response of masu salmon in different 3 minute anesthesia treatments: (A)50 mg/L clove oil; (B)100 mg/L MS-222; and (C) 100 mg/L MS-222 + 100 mg/L $NaHCO_3$. The values are means ± SE of data obtained from 3 fish each.

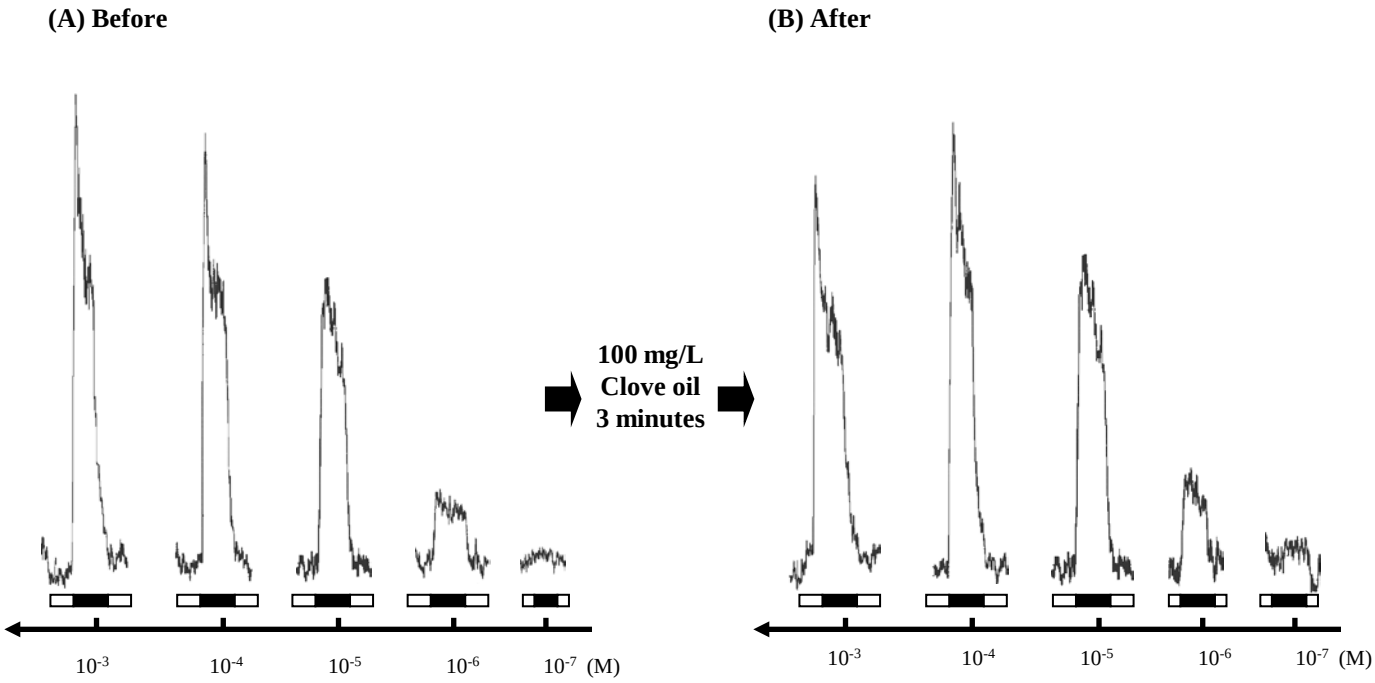

(A) Before
(B) After
100 mg/L
Clove oil
3 minutes
10-3
10-4
10-5
10-6
10-7 (M)
10-3
10-4
10-5
10-6
10-7 (M)

Fig.1a.

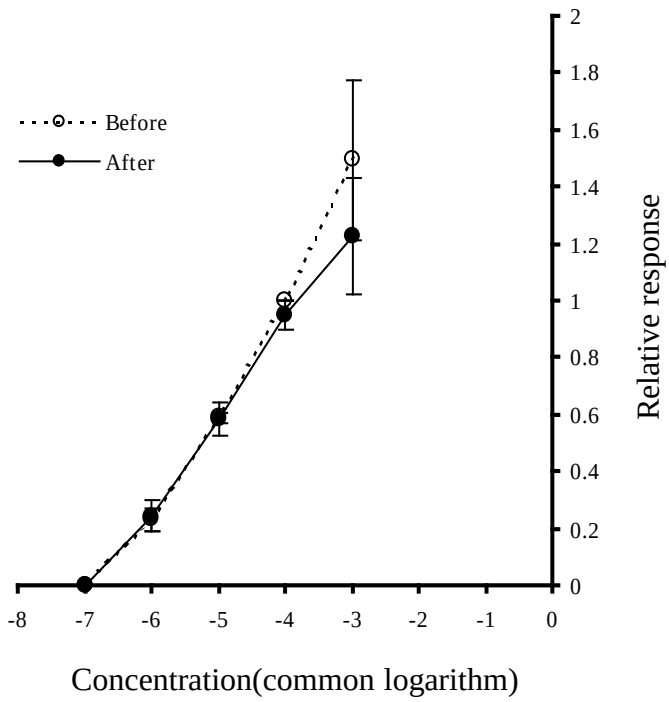

Before
After
Relative response
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-8
-7
-6
-5
-4
-3
-2
-1
0
Concentration(common logarithm)

Fig.1b.

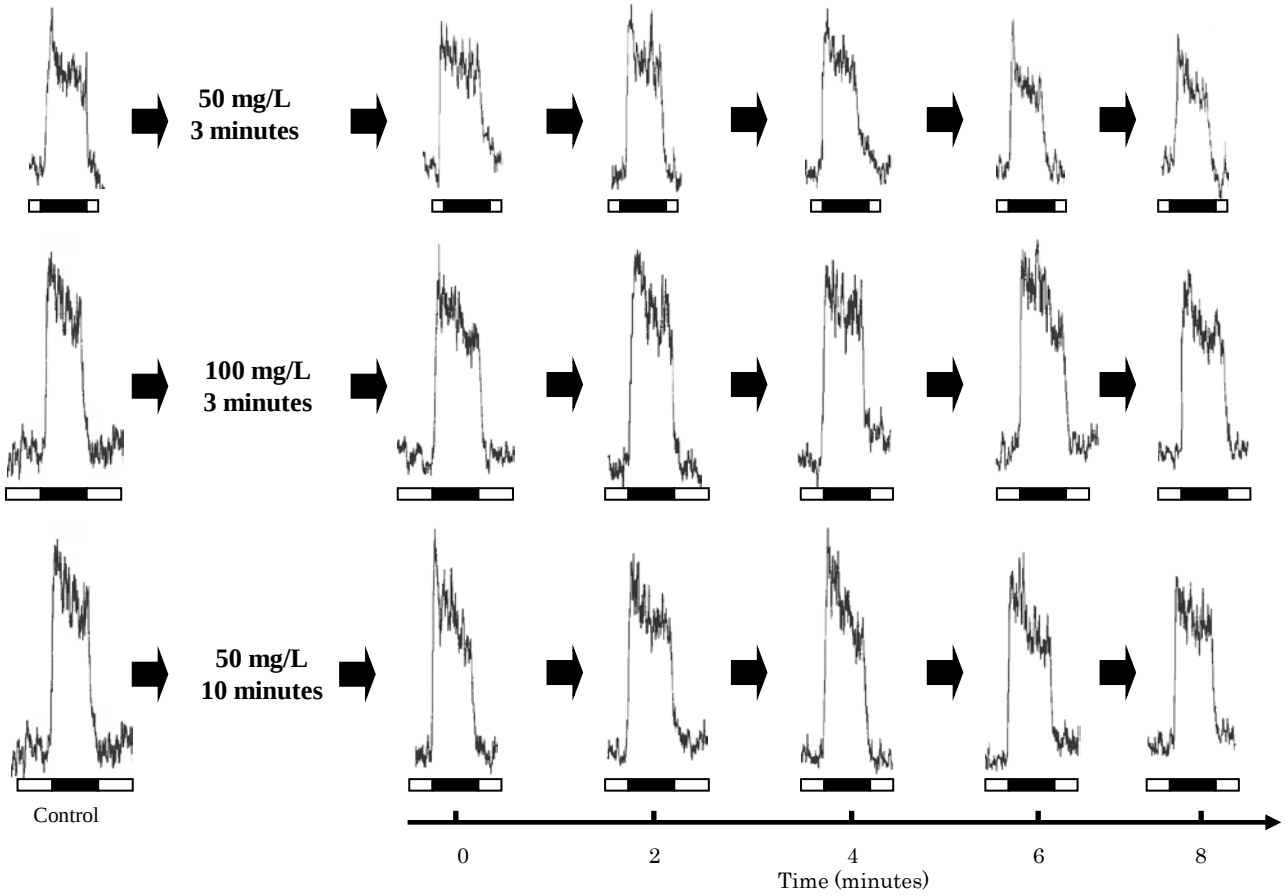

50 mg/L
3 minutes
100 mg/L
3 minutes
50 mg/L
10 minutes
Control
0
2
4
6
8
Time (minutes)

Fig.2a.

n: :number of examined fish

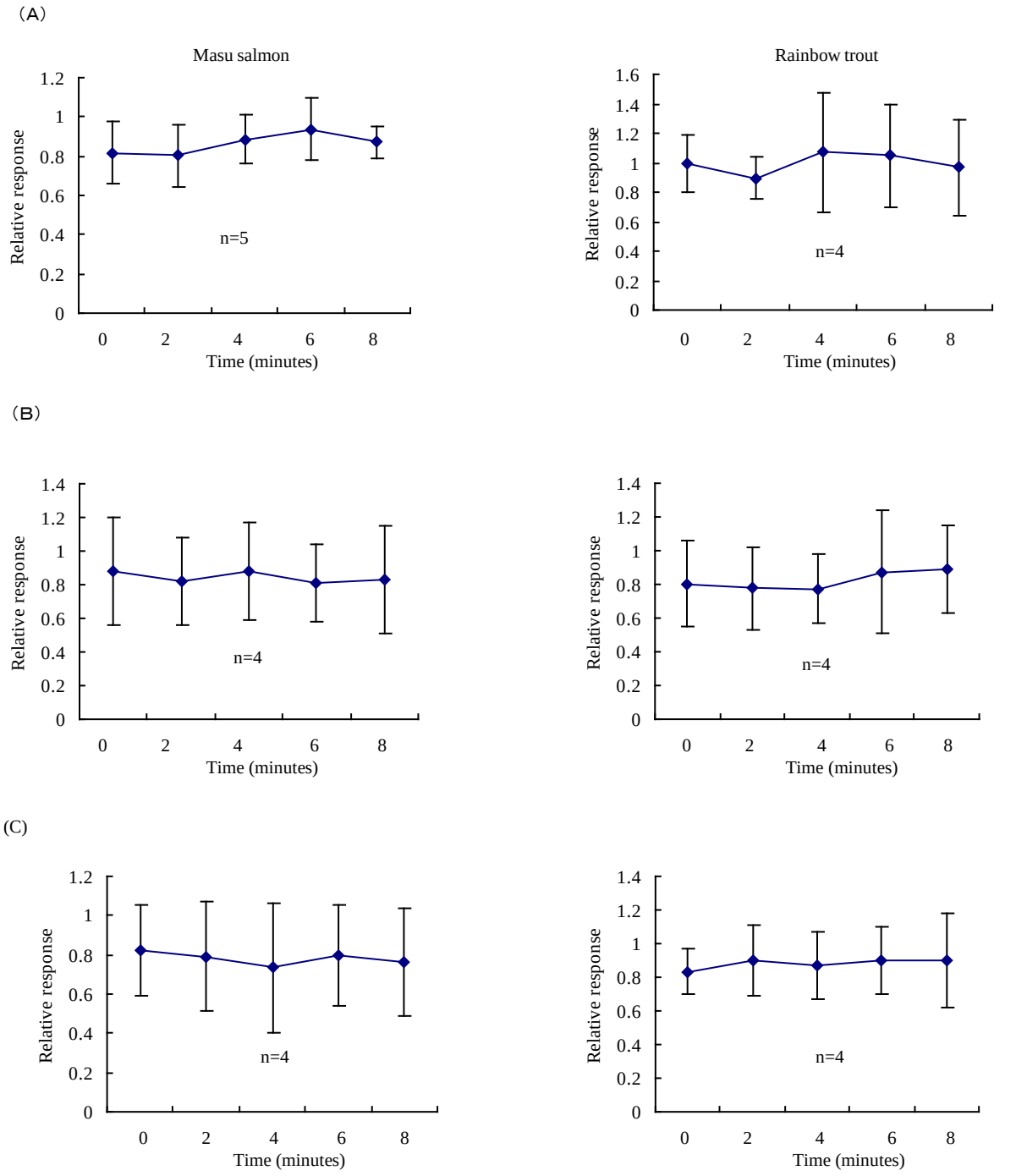


Fig.2b.

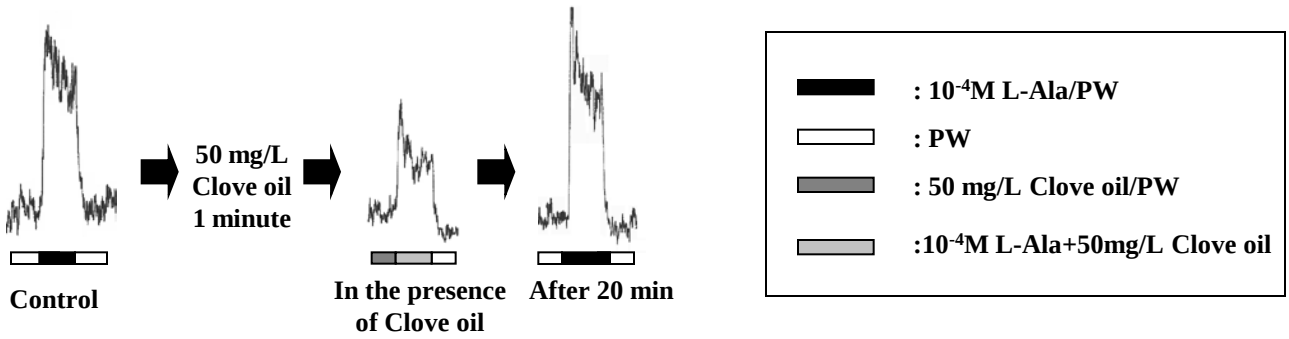

50 mg/L
Clove oil
1 minute
Control
In the presence
of Clove oil
After 20 min
: 10-4M L-Ala/PW
: PW
: 50 mg/L Clove oil/PW
:10-4M L-Ala+50mg/L Clove oil

Fig.3a.

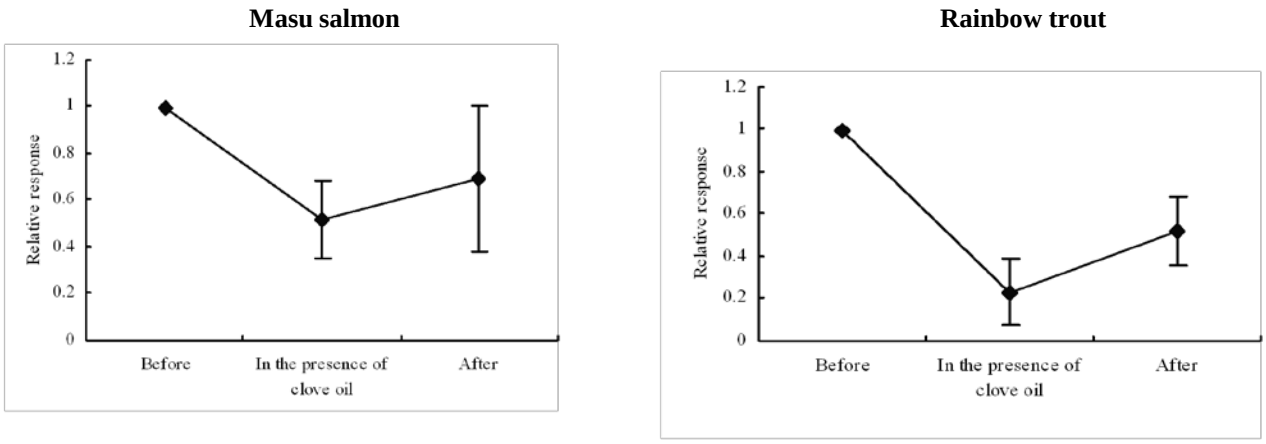

Masu salmon
Rainbow trout
Relative response
1.2
1
0.8
0.6
0.4
0.2
0
Before
In the presence of clove oil
After

Fig.3b.

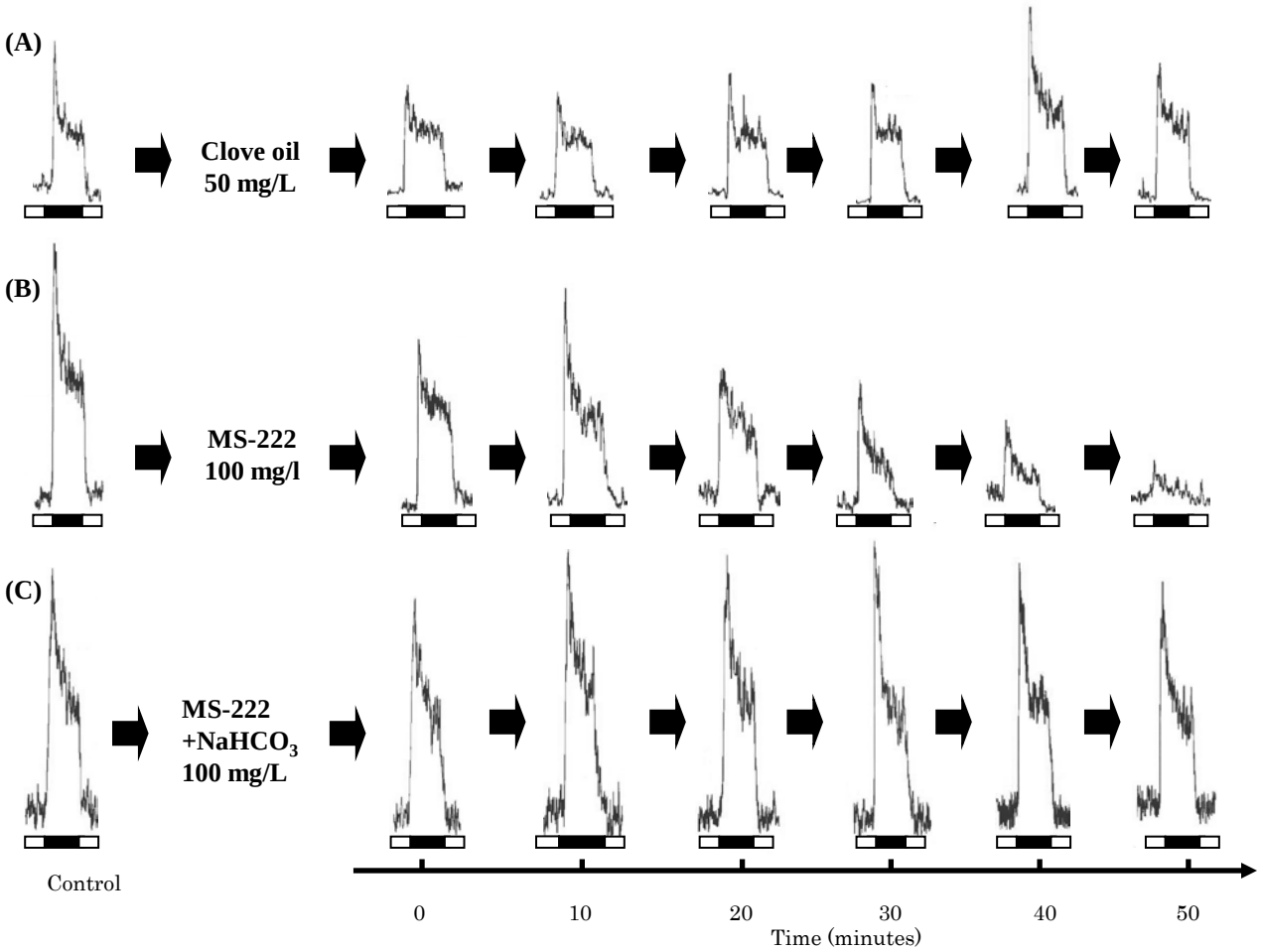
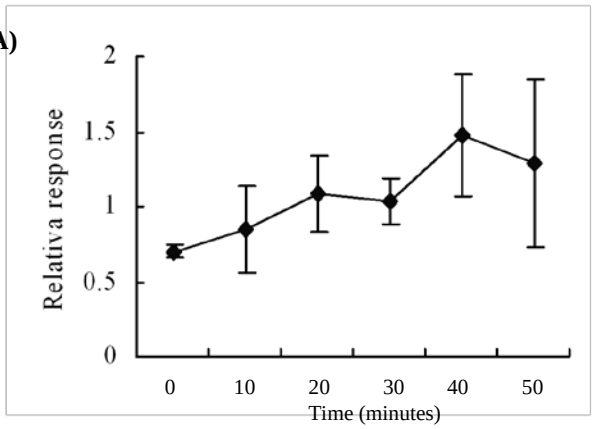

(A)
Clove oil
50 mg/L
(B)
MS-222
100 mg/l
(C)
MS-222
+NaHCO3
100 mg/L
Control
0
10
20
30
40
50
Time (minutes)

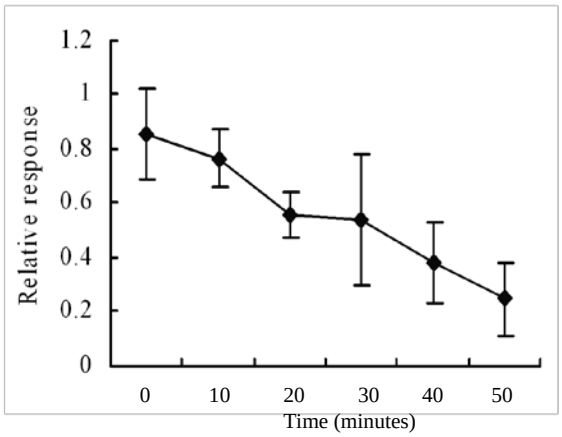
Fig.4a.

(A)



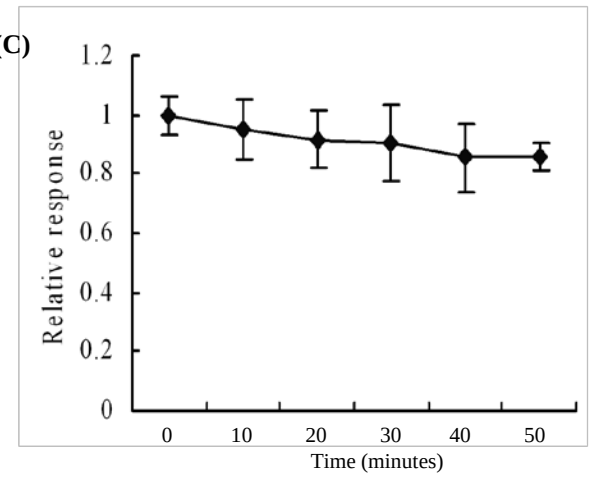
Clove oil

(B)



MS-222

(C)



MS-222+$NaHCO_3$

Fig.4b.