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ORIGINAL RESEARCH

A noninvasive monitoring device for anesthetics in fish

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Abstract: A noninvasive device capable of recording both gill and lateral fin movements was assembled and used to analyze initial and post-treatment activity frequency (Hz) in fish exposed to anesthetics. Exposure of platy fish (*Xiphosphorus maculatus*) to saponins from quillaja bark (0.185 mM and 0.555 mM) initially caused hyperactivity, but within five minutes all activity ceased and the fish failed to recover. In contrast, clove oil (67 µg/L) added to water at 22°C reduced activity by 22.8% ± 8.9% ($P = 0.038$) after 125 ± 19 sec, a sedative effect that was totally reversible. Cinnamon oil compared with clove oil had a significantly longer time to sedation (125 ± 19 versus 235 ± 24 sec, $P = 0.02$), although no significant difference in the decline in activity was noted.

Keywords: anesthetics, cinnamon oil, clove oil, platy fish, recording device, teleost

Introduction

It is generally accepted that anesthesia may be a useful way to maintain fish stress- and injury-free during essential manipulations in aquaculture.[1] Quite a number of anesthetics have been used or evaluated for use in aquaculture, but issues such as toxicity, safety, and cost are frequently limiting factors.[1–6] Increased concern about animal welfare and potential suffering caused to aquaculture fish during routine manipulations (eg, handling, medication, and transport) has intensified the search for "good" anesthetics.[7,8]

Characterization of the efficacy of anesthetics has generally been carried out by exposure experiments and visual monitoring of the time to sedation and time to recovery, something that could perhaps be mechanized. A range of such studies exist in aquaculture species, such as sea bream (*Sparus auratus*), white sea bream (*Diplodus sargus*), sharp snout sea bream (*Diplodus puntazzo*), sea bass, rock bream (*Oplegnathus fasciatus*), Senegalese sole (*Solea senegalensis*), and salmon.[9–15] These studies reveal that environmental factors, such as water salinity and temperature, and species-specific susceptibility, influence efficacy of anesthetics.

Plant extracts represent a potential source of new anesthetics and have long been used by indigenous tribes of South America and almost every other continent as part of their arsenal of fishing tools. Common toxic piscicides of plant origin include sesquiterpenoids, furanocoumarins, diterpenoids, quinines, and triterpenes. However, the most commonly used piscicides are rotenoids and saponins. Rotenone is known to inhibit NADH-Q reductase in the mitochondrial electron transport chain, and this prevents the mitochondria from using NADH as a substrate. Electron transfer

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is virtually halted, and the organism cannot produce an adequate supply of adenosine triphosphate, which results in asphyxia and paralysis, followed by death.[16] An anesthetic which has become popular in recent years is the extract of *Eugenia caryophyllata*, or clove oil, which contains eugenol (2-methoxy-4-2-propenyl phenol), a very effective anesthetic for fish and considered by the US Food and Drug Administration as a generally recognized as safe (GRAS) compound. Cinnamon bark (*Cinnamomum zeylanicum*) also contains eugenol, but its use as an anesthetic has not been explored.

Thus, the objective of the present study was firstly to develop a noninvasive and automated monitoring device to assess anesthesia in fish, and then subsequently use this device to establish the potential mode of action and identification of new anesthetics for use in fish. To this end, the anesthetic potential of saponins and volatile oils from clove and cinnamon has been determined using the platy fish (*Xiphosphorus maculatus*).

Material and methods

Fish

Adult platy fish, 6–8 cm in length, were purchased from a pet shop and transported in plastic bags insulated with paper to the experimental facility (Section for Physiology and Biochemistry, Faculty of Life Sciences, Copenhagen University). Fish (n = 6/tank) were acclimated to 10 L glass containers containing dechlorinated tap water at 22°C and a photoperiod of approximately 16 hours light and eight hours dark (May in Denmark, sunrise 04:45 hours and sunset 21:30 hours) and allowed a 48-hour period to recover from the stress of transportation and handling. The water had a documented quality comprising oxygen 10.7 mg/L, an iron content of 0.006 mg/L, pH 7.6, and a conductivity of 66.3 mS/m. Tanks were cleaned daily and 50% of tank water substituted every three days. The fish were fed twice daily to satiety with TetraMin® (Tetra GmbH, Melle, Germany) comprising crude protein 48%, crude fat 8%, crude fiber 2%, ash 11%, moisture content 6%, and vitamins. Experiments were carried out during May 2007.

The study was conducted in compliance with the European Community guidelines (86/609/EU) concerning the protection of experimental animals, and in accordance with local national guidelines for animal experimentation.

Activity measurements

A noninvasive recording device, comprising three copper electrodes, was assembled in a 300 mL glass tank in order to monitor and record fish activity accurately (see Figure 1). The device was capable of recording both gill and lateral fin movements, which were then analyzed in terms of initial and post-treatment activity frequency (Hz). Activity signals were recorded via an ML 132 amplifier connected to a ML 780 PowerLab/8S A/D converter connected to an iBook G4 running Chart5 v.5.4 Software (AD Instruments, Australia). The data recording was at a sampling speed of 40,000 data samples per second (40 kHz) and the input impedance of the amplifier was 200 mΩ differential. Decline in activity was defined as being either or both a reduction in signal amplitude (mV, constituting less powerful fin and gill movements), or signal frequency (Hz, constituting an increase in time between repeated movements of fins and gills).

Clove, cinnamon oil, and saponins

Commercially available cinnamon and clove oils were used in this study (Urtegaarden Aps, Allingåbro, Denmark), and their action was compared with that of saponins. Clove oil extract imported from Indonesia, was derived from the flowering buds of *E. caryophyllata*, and was 100% pure. Cinnamon oil extract imported from China, was derived from the bark of *C. cassia*, and was also 100% pure. A standard solution was prepared of purified saponin from quillaja bark (1 mg/mL distilled water; Sigma-Aldrich, Denmark).

Analytical procedure

Recording of activity was carried out in a 300 mL volume tank containing 150 mL of water at a constant temperature (22°C). Preliminary experiments were carried out to optimize the setup of the recording system and to establish an approximate dose range of clove and cinnamon oil appropriate for sedation of platy fish (only opercula movements visible). The initial experiments linked results of instrument recordings to visually evaluated states of anesthesia in the fish, ie, partial loss of equilibrium and erratic swimming, mild sedation (slowed opercula, and anal fin and tail fin movement), and sedation (very slow/irregular opercula and fin movements).[1] Recovery time was evaluated as being the time taken to recover normal swimming and responsiveness to visual stimuli upon removal to a recovery tank containing clean water. Food was withheld from the fish on the day of the experiment.

For studies of the anesthetics, fish were placed in the recording system and given time to settle (approximately five minutes) before initiating recording. Activity was recorded for approximately one minute prior to adding the test anesthetic, which was prepared fresh in 5 mL of

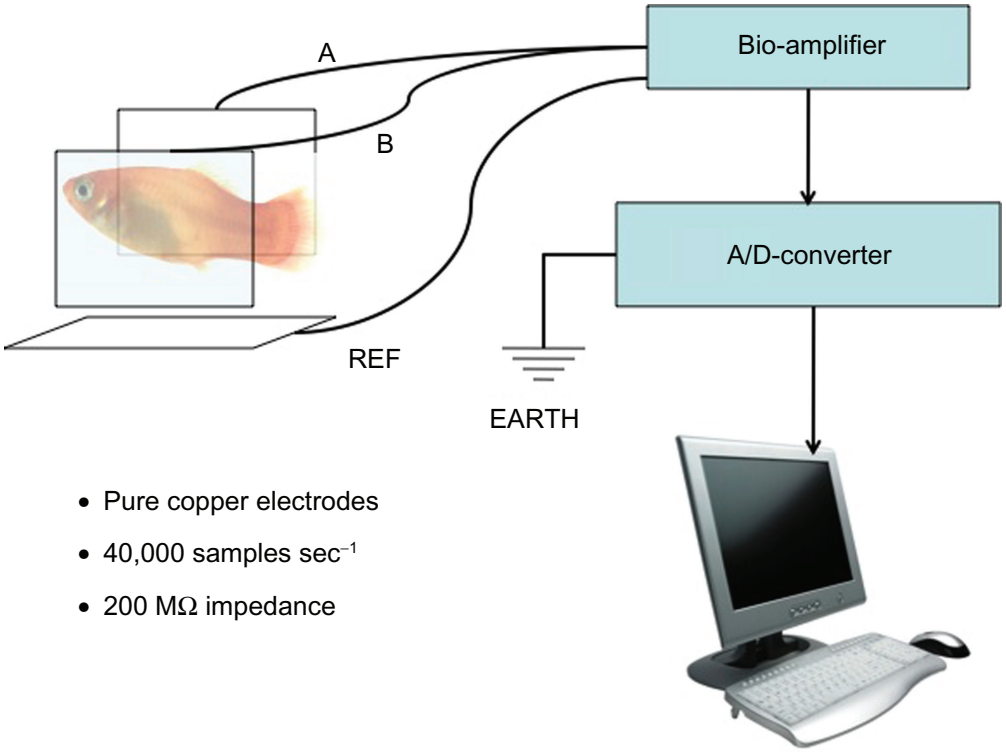

Figure 1 A schematic of the noninvasive recording device used to record both gill and lateral fin movements in platy fish. The pure copper electrode (A, B, and reference) were connected to a ML 132 Bioamplifier, connected to a ML 780 PowerLab/8S A/D converter, connected to an iBook G4 running Chart 5 vs 5.4 Software (AD Instruments, Australia). Data collection was achieved by means of a double-differential array, in which the signal from electrode B was subtracted from that obtained from electrode A, before finally being corrected for any "background" noise within the system, sensed through the third reference electrode. The data recording was at a sampling speed of 40,000 data samples per second (40 kHz), giving very smooth and precise measurements of individual "spikes/movements", and the input impedance of the amplifier was 200 mΩ differential.

water at 22°C and vigorously mixed immediately prior to experimentation. To minimize disturbance of fish, the anesthetic (final concentration: clove oil 0.67 µg, 6.7 µg, 33.5 µg, or 67 µg/L, or cinnamon oil 0.67 µg, 6.7 µg, 33.5 µg, or 67 µg/ L was added to one corner of the experimental tank containing 145 mL of dechlorinated tap water using a 5 mL pipette and mixed by rapidly pipetting up and down. Evaluation of the anesthetic effect of purified saponin from quillaja bark was carried out by adding 15, 50, and 150 mg dissolved in 5 mL of distilled water to the experimental tank (150 mL volume, ie, approximately 0.055 mM, 0.185 mM, and 0.555 mM, respectively) and mixing vigorously as described above.

To assess the possible site of action of clove oil, a second group of experiments was carried out with Ruthenium red ($Ru_2OH_2Cl_4.7NH_3.3H_2O$, Number 12319, 99% purity; Merck, Denmark), a noncompetitive vanillin receptor antagonist, at a final concentration of 15 µM. Fish were pre-exposed in the experimental circuit to Ruthenium red for five minutes before addition of the test anesthetic. Fish activity was recorded prior to and during exposure to Ruthenium red, and during subsequent exposure to test anesthetics.

Statistics

Data are presented as mean ± standard error of measurement (SEM). Differences between means were tested for statistical significance with the use of GraphPad Instat 3 for Mac (version 3.0b, 2003; GraphPad Inc., La Jolla, CA), with an additional test for Gaussian normal distribution. Data that were normally distributed according to a Kolmogorov and Smirnov test, and having an equal variance, were tested for significant difference between means using a *t*-test. Otherwise a Mann–Whitney nonparametric analysis was performed. Differences showing a P value < 0.05 were considered significant (Table 1).

Results

Recording device

The system used for recording fish activity (defined as being opercula movements as well as fin movements), proved very sensitive in terms of its ability to record both unipolar and bipolar signals (see Figure 2). Typically, background electrical noise was in the order of magnitude of 0–2 µV (amplitude of oscillating signal), whilst extremely small opercula and fin movements yielded a signal with an amplitude of at least 10 µV. Indeed, usually the recordings had

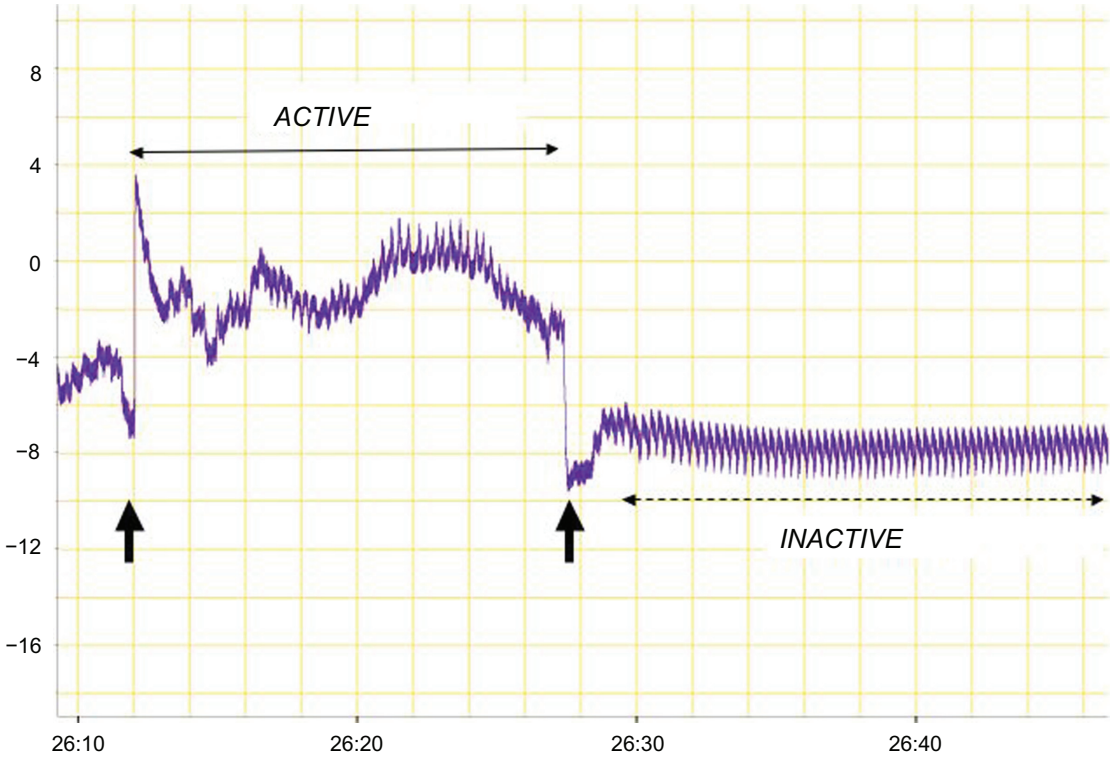

Figure 2 A typical recording of both gill and lateral fin movements, which were then analyzed in terms of the initial and post-treatment activity frequency (Hz) of platy fish. Note that the recordings denoted by ↑ indicate an 180° turn by the platy fish within the recording chamber. The region of the recording denoted by a solid two-arrow line represents a period of active movement by the fish (unstable baseline and irregular spikes), whilst the region denoted by the dotted two-arrow line represents a stable period of inactivity measuring gill and fin activity (3.61 Hz) alone. The y-axis = activity (mV) and x-axis = time (sec).

an amplitude of 23–26 μV, giving a signal to noise ratio of 11.5:1–13:1.

Analytical procedure

Saponin

Saponin from quillaja bark (0.185 mM and 0.555 mM) was tested on platy fish (n = 4–6) and initially caused hyperactivity, but within five minutes of exposure all activity ceased and fish died very suddenly. All of the concentrations tested caused death and no further studies were carried out.

Clove and cinnamon oils

Clove oil (33.5 or 67 μg/L) had a rapid action and totally reversible effect, and sedated fish within two minutes of exposure. Activity, as determined by fin movements, gave a good index of anesthetic effect, and dropped significantly (22.8% ± 8.9% decline, $P = 0.038$) after 125 ± 19 sec in fish exposed to clove oil. An additional dilution of clove oil resulted in a significantly longer time to sedation (125 ± 19 versus 530 ± 33 sec for 33.5 and 67 mg/L versus 6.7 μg/L, respectively, $P = 0.004$), without any further significant decline in activity. Further dilutions of clove oil (0.67 μg/L) caused an initial period of hyperactivity, which was followed by reduced activity and fish never reached a state of total sedation.

Cinnamon oil (67 μg/L) also had a fairly rapid action and totally reversible effect and sedated fish within four minutes of exposure. After preliminary testing (0.67–67 μg/L) in order to optimize the concentration of cinnamon oil needed to produce reversible anesthesia, a comparison of clove oil (67 μg/L) versus cinnamon oil (67 μg/L) revealed a significantly longer time to sedation (125 ± 19 versus 235 ± 24 sec, $P = 0.02$), although no significant difference in decline in activity was noted. Finally, cinnamon oil was noted to cause an initial period of hyperactivity, which was followed by reduced activity, similar to that found with low concentrations of clove oil (0.67 μg/L).

Ruthenium red and clove oil

Incubation of platy fish for a relatively short period of four minutes in a 15 μM Ruthenium red aqueous solution had no detrimental effect on the anesthetic effect of 67 μg/L clove oil in water, because platy fish were completely motionless after approximately 125 seconds. After a much longer 30 minute incubation of platy fish in a 15 μM Ruthenium red aqueous solution, no adverse effects were noted. On addition

Table 1 Summary of results, highlighting the type of compound tested, the concentration applied, the time recorded to sedation, reversibility, and any statistically significant difference between individual compounds and concentrations*

Compound	Concentration	Time to sedation (sec)	Reversibility	Statistical significance
A: Quillaja bark saponin	0.185 mM	<300	No – death	N/A
A: Quillaja bark saponin	0.555 mM	<300	No – death	N/A
B: Clove oil	33.5 μg/L	125	Yes	N/A
B: Clove oil	67.0 μg/L	125	Yes	NS versus 33.5 μg/L
B: Clove oil	6.7 μg/L	530	Yes	$P = 0.004$ versus 33.5 μg/L and 67.0 μg/L clove oil
B: Clove oil	0.67 μg/L	No sedation	Yes	N/A
C: Cinnamon oil	67.0 μg/L	<240	Yes	$P = 0.02$ versus 67.0 μg/L clove oil
C: Cinnamon oil	0.67 μg/L	No sedation	Yes	N/A
D: Ruthenium red	15 μM for 30 min with 6.7 μg/L clove oil	No sedation at 900	Yes	N/A

Note: *For details of statistical tests see Materials and methods section.
Abbreviation: N/A, not applicable.

of clove oil (67 μg/L), full anesthesia was achieved within 290 seconds. However, in a repeat experiment in which a lower dose of clove oil was administered (6.7 μg/L) and a similar 30-minute equilibration period in Ruthenium red 15 μM was used, and no signs of anesthesia were noted even after 15 minutes.

Discussion

This study, which is the first to design and test a noninvasive device capable of recording both gill and lateral fin movements, has examined the efficacy of a number of natural anesthetics on platy fish.

Recording device

The choice of copper electrodes in this recording system gave a much cleaner signal in terms of background noise than that obtained using stainless steel electrodes (data not shown). However, a far cleaner and more sensitive recording device could be assembled if 24 carat gold or pure platinum electrodes had been used. The use of such "noble metals" as electrodes would also facilitate the application of this device with salt-water fish. Moreover, the flat nature of the recording electrodes lends itself to use with flatfish, provided an electrode were placed below and above the fish, and contact between the fish and the electrodes were prevented by means of a plastic net or gauze.

Secondary plant metabolites as piscine anesthetics

Plant poisons, which are often saponin-based, are still in use by indigenous tribes in many places in the world today. Furthermore, reports indicate that whilst saponins can be powerful poisons, they are not usually fatal, and fish that are washed in untainted water often revive fully and return to their pretoxin condition.[17] One of the ways in which saponins act is via hemolysis of red blood cells in the fish, and in the present study this occurred slowly (results not shown), suggesting that impairment of respiratory capacity in exposed fish might be expected to be slight. It seems probable that saponins "stunned" fish rather than anesthetized them, a proposal supported by reports that fishermen using saponins need to gather fish quickly as they float to the surface because they recover rapidly.[17] Their results indicate that saponins and related compounds can be excluded from the list of potential new anesthetics, although their role as a calmative agent may warrant closer investigation, particularly as they are reported to have no adverse effect on the flavor or edibility of the fish.

Clove bud oil comprises a number of volatile oils, totaling 15%–18% v/w. Of these volatile oils, eugenol represents some 80%–90%, eugenyl acetate represents 2%–27%, and β-caryophyllene represents 5%–12%. In contrast, cinnamon bark comprises less volatile oil, totaling approximately 4% v/w. Of these volatile oils, cinnamaldehyde represents some 60%–75% and phenols represent 4%–10%, of which eugenol, methyl eugenol, and safrole are components. Of the various types of cinnamon bark, the oil of *C. zeylanicum* is stated to contain the highest amount of eugenol. Moreover, cinnamon leaf oil is reported to contain far higher concentrations of eugenol, ie, 80%–96%, depending on species.

At present we are unable to explain the difference in time to sedation with cinnamon versus clove oil, although it may be

due to differences in eugenol content, or other as yet unidentified active components of the oils. A closer investigation of these two oils in terms of their composition is now required if this particular observation is to be explained. In addition, future studies should also focus on the degree to which these oils can be used repeatedly to sedate fish, as previous studies have noted that desensitization to eugenol and/or other compounds within the oils occurs over time.[9] The dose of cinnamon or clove oil required for anesthesia in the present study was significantly lower than those reported in previous studies (range 4–150 mg/L) using aquaculture species, but this is probably a consequence of the anesthetic endpoint chosen, species, water temperature, and other factors previously described to influence anesthesia.[9,10,12–15,18–21]

Potential mode of action of eugenol

Eugenol, the major component of the essential oils of clove and cinnamon, has been used to relieve pain arising from a variety of sources.[22] Eugenol ($C_{10}H_{12}O_2$) is an allyl chain-substituted guaiacol which acts in both the pre- and postsynaptic sites of neurons by blocking the Ca^{2+} current, decreasing membrane potential, and decreasing gamma aminobutyric acid, acetylcholine, and glutamate-evoked excitatory responses at submillimolar concentrations.[23] The fact that eugenol, as a natural capsaicin congener, also contains vanillin-moiety-like capsaicin suggests that eugenol may act on vanillin receptor 1 (a transient receptor potential ion family channel), as capsaicin does in sensory neurons.[24–26] In the present study, the rather incomplete effect of the vanillin receptor 1 noncompetitive antagonist, Ruthenium red, suggests that other receptors are also affected by eugenol. It is therefore important to determine whether a high concentration of eugenol is capable of acting via other receptors, with a "poorer fit" pharmacologically, to impair the effectiveness of Ruthenium red.

Conclusion

It is concluded that the recording device assembled in this study can be used successfully to record anesthetic effects on platy fish, and indeed could be readily adapted to measure anesthetic effects in juvenile aquaculture species too. The results of the present study indicate that saponins are not effective as a fish anesthetic but that clove oil, as previously reported, and cinnamon oil, offer a safe alternative to current anesthetics, sedating fish in a reversible fashion within 125 ± 19 sec and 235 ± 24 sec for clove and cinnamon oils, respectively. Moreover, studies with Ruthenium red suggest that part of the anesthetic effect of clove oil is through the vanillin receptor 1, but that other mechanisms are also involved. It is therefore proposed that the recording device developed be adopted as a reliable, rapid, and noninvasive method for monitoring the efficacy of anesthetics in fish where other monitoring systems are too labor-intensive or unsuitable.

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