Improving welfare in fish requires avoiding pain, stress and suffering. Propofol, 2,6-diisopropylphenol, seems to be a good candidate as a fish anaesthetic, however, no study regarding propofol influence on Nile tilapia has yet been reported. With this aim, the efficiency of propofol and benzocaine was compared as anesthetic for fish following immersion exposure. Nile tilapia (*Oreochromis niloticus*) was used as model due its importance in aquaculture, been the most important fish for human consumption, where 4.5 million tonnes of fish are produced worldwide. At first, determination of effective anaesthetic concentrations to induce complete anesthesia was determined, under immersion, considering time to start decubitus stage. Then the magnitude of these anesthetics was tested, measuring its effects on time remaining in decubitus, posture recovery, ventilatory frequency (VF) and latency to feed. Benzocaine induced reduction of VF under decubitus. After the anesthetic effects, VF returned quickly to basal levels. The same pattern was observed for propofol, however with no return to basal levels after recovery. Time to start decubitus was similar in both anesthetic, but time to return was higher in propofol. The latency to feed was longer in fishes submitted to propofol. Thus, propofol is a more powerful anesthetic than benzocaine in Nile tilapia, with longer duration and deeper effect. Although the common usage of propofol is by intravenous injection, here we show that immersion is efficient as an anesthetic in fish and could be adopted as a protocol in experimentation as well aquaculture management. Analgesia in fish is an area in need of significant research as only a few studies exist and they provide some contrasting results.

**Keywords** Animal welfare . Aquaculture . Management . *Oreochromis niloticus*. Ventilatory frequency . Anesthesia

**Introduction**

Animal welfare is an increasing concern for scientists and public in general. This concern includes many organisms, from vertebrates to invertebrates. In this context, anesthesia usage in fishes has become an increasing necessity. While MS222 and Benzocaine are the most commonly used anesthetics for fish, other alternatives have been explored to improve this process (Grimm et al. 2015).

The purpose of an anesthetic is to avoid pain and mitigate stress despite it can activate the stress response (Bolasina 2006). According to Zahl et al. (2012), there is a reduction of welfare with these side effects of anesthetic, such as osmotic stress and respiratory acidosis. Therefore, it is necessary to be cautious when using these chemicals. Beside this, variables such as intensity of stressor, fish species, developmental stage and environmental conditions may affect the efficacy of anesthetic (King et al. 2005).

Strong-effect anesthetics, with profound action on central nervous system (CNS) can be desirable since
they have a fast way of action. The anesthetic commonly used in fish through immersion is benzocaine (Gomes et al. 2001), with a peripheral action on brain, making the anesthetic process slower, leading the animal to experienced discomfort and can lead to tachycardia, increase respiration and lead to hyperglycemia (Neiffer and Stamper 2009). Propofol (2,6-diisopropylphenol), on the other hand, is a general anesthetic that acts quickly on CNS. Its mechanisms involve the modulation of inhibitory activity of GABA receptors (Sonner et al. 2003). At intravenous injection, propofol has a fast distribution throughout CNS because of its high lipid solubility. The recovery is fast and soft, making propofol a different anesthetic from traditional ones as MS-222 (Gressler et al. 2012).

In fish, intravenous injection of Propofol can cause injuries and pain (Fleming et al. 2003). Furthermore, this is not a recommended way to anesthetize fish because of immobilization requirements, making it a difficult process to anesthetize small individuals and impractical for a large number of specimen. Further, out-of-water manipulation requires usage of anesthetics first in order to maintain animal welfare. Therefore, anesthesia by immersion is logical in fish experiments and procedures.

Practices that ensure better life quality for bred animals are directly related to sustainability of production and to the perceptions of sustainable fish farming. In aquaculture, Oreochromis niloticus (Linnaeus, 1758) (Nile tilapia) is one of the most important fish for human consumption; reaching 4.5 million tons produced worldwide (Fao 2018). With the growth of fish production and consumer awareness about the techniques applied to this production, welfare requirements that ensure the fish wellbeing stand out as an important contribution to the aquaculture area, aiming at the creation of production patterns that expose fish to minimal stress and less imbalance to the ecosystem. Practices that guarantee welfare are already applied to other production areas, such as cattle, swine, and poultry, for example. However, when it comes to aquatic animal production, the application of welfare precepts is still poor.

Fish display robust neuroendocrine and physiologic stress responses to noxious stimuli. Many anesthetic, sedative, or analgesic drugs used in other vertebrates reduce stress in fish, decrease handling trauma, minimize movement and physiologic changes in response to nociceptive stimuli, and can be used for euthanasia. But extrapolating from limited published anesthetic and sedative data to all fish species is potentially harmful because of marked anatomic, physiologic, and behavioral variations; instead, a stepwise approach to anesthetizing or sedating unfamiliar species or using unproven drugs for familiar species is advisable.

Since propofol has been poorly studied in fish and never tested in Nile tilapia by immersion, the objective was to test the efficiency of anesthetic propofol immersion in Nile tilapia, comparing with benzocaine, the most used anesthetic in fish. Nile tilapia was used as an animal model because of its high production in aquaculture and its cosmopolitan characteristic. We hypothesis that propofol can be a reliable alternative in fish anesthesia for unconscious induction.

**Materials and methods**

**General conditions**

Juveniles of Nile tilapia, Oreochromis niloticus, hatched and grown in fish farm ponds were acclimated for 30 days (in 500L circular tanks; 6 fish/L) before the experiment. The temperature was set at 28-30 °C and photoperiod at 12-h L: D. Continuous aeration through a biofilter was provided by a re-circulating system that included small pieces of PVC tubing that provided shelter for the fish. The fish were fed twice a day (09:00 and 14:00) and the tank was siphoned when necessary. Nitrite and ammonia levels were kept under 0.05mg/L and 0.5 mg/L respectively, to maintain water quality. Fish were with healthy parameters such as proper feeding behavior, food ingestion, normal locomotors patters, normal coloration and skin condition.

**Experimental design**

To test the efficiency of anesthetic propofol in fish by immersion, our strategy consists basically in measure physiological features such as ventilatory frequency (VF) and latency for food ingestion as surrogate to unconscious and recovery from it. First, the basal levels of these parameters were measured. Then we submit individual fish to the tested anesthetics benzocaine and propofol in five concentrations, with eight replications each. Minimal doses of the anesthetics were firstly determined and secondly the effect of
propofol and benzocaine in VF and latency to feed were analyzed. The experiment design is expressed in Figure 1. All procedures were approved by Ethical Committee for Animal Research of São Paulo State University (UNESP), Campus Botucatu (CEUA – protocol#125).

Concentrations of benzocaine and propofol

Fish were food deprived for 24 hours before the beginning of experiments. They were then transferred to 2L aquariums with constant aeration, temperature 24.5±0.16 °C, luminosity 400 lux, nitrite <0.05 mg L⁻¹, ammonia <0.05 mg L⁻¹, pH=7±0.2 and water properly saturated with oxygen.

Benzocaine (ethyl p-aminobenzoate 80 mg L⁻¹ Sigma-Aldrich PubChem Substance ID 24894416) and propofol (2,6 diisopropylphenol; Diprivan 1%; Astra Zeneca, lot X09046B) were used as anesthetics. To determine the minimum dose of each anesthetic, benzocaine started at 6.5 mL L⁻¹ and two doses below and two above this threshold were used (1.5, 3.25, 6.5, 13 and 26 mL L⁻¹). As propofol did not have this reference for teleost fishes by immersion, 0.3 mL L⁻¹ was obtained through a pilot study, considering time of decubitus as showed by individuals under benzocaine anesthetic effect. Therefore, the following concentrations for Propofol: 0.075, 0.15, 0.3, 0.6 and 1.2 mL L⁻¹ were determined and posture pattern (lateral decubitus and posture recovery) were measured as signals of anesthesia. We tested eight fish in each concentration, either for benzocaine or propofol, with a total of 80 fish. Fish weight average and standard deviation: 22.85 ± 2.57 g and length average and standard deviation: 8.80 ± 0.4 cm. There was no difference between groups in weight and length, P< 0.05.

Specifically, the anesthesia procedure was as following: at the end of two hours of acclimation in the aquarium without anesthetic, each fish were individually transferred to another aquarium with anesthetic (benzocaine or propofol) and after four minutes of lateral decubitus, fish were transferred back to the initial aquarium, without anesthetic, for recovery. All aquariums were supplied with constant aeration. VF was measured at five time points moments: 1) immediately before the transfer to the aquarium containing anesthetic (Basal measure); 2) at the moment that fish started the decubitus process; 3) immediately after transfer back to initial aquarium (without anesthetic); 4) the moment that fish returns to its normal posture; and 5) four minutes after return to normal posture (rest). VF was measured counting the number of operculum openings. We measured the time required for 20 opercular beats and calculated the number of opercular beats per minute for each evaluated fish. Fish were killed with overdose of benzocaine after the experimental procedures.

Anesthetic action: ventilatory frequency

Same procedures as before, but 6.5 mL L⁻¹ for benzocaine and 0.3 mL L⁻¹ of propofol concentrations were applied (because anesthesia effects start at these concentrations) and a control group, without any anesthetic. VF was measured every minute, the latency for decubitus (anesthesia induction) and return to normal posture (recovery). 15 fish were used for each anesthetic and 15 fish for control group (20.07 ± 2.57g and 8.28 ± 0.35cm).

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Fig. 1 Experimental study design
Latency to feed

All fish were held in isolated aquariums (30 x 20 x 10cm) for five days, fed daily using food for tropical fishes (1mg/fish) at 9:00am. On the sixth day, fish were transferred individually to aquariums with the same dimensions, containing one type of anesthetic (benzocaine 6.5 mL L⁻¹ or propofol 0.3 mL L⁻¹). The same conditions for control group were applied but with no contact with anesthetic. When fish entered the decubitus stage, they were immediately transferred to the initial aquarium. Food was offered (1 mg fish⁻¹) and latency to feed was measured; n= 12 replicates for each anesthetic and 12 fishes as control group (21.52 ± 1.11g and 8.66 ± 0.25cm).

Statistical analysis

Repeated measure Analysis of Variance (ANOVA) was used to compare VF, since each individual was measured multiple times. Time to induce decubitus and recovery from anesthesia and latency to feed on different doses of each anesthetic was assessed through ANOVA one-way. Significance level was set at α=0.05.

Results

We used anesthetic concentrations that all fish entered decubitus stage. For benzocaine, this concentration was 6.5 mL L⁻¹, inducing decubitus in 4’26’’ ± 1’09’’. For propofol concentration, 0.3 mL L⁻¹ induced decubitus in 4’28’’ ± 1’23’’ (Figure 2).

Fish did not show any sign of decubitus and there was no difference in VF between basal level and following exposure at the lowest concentrations (benzocaine: 1.625 mL L⁻¹ and propofol: 0.075 mL L⁻¹; P>0.05). At the second lowest concentrations, benzocaine (3.25 mL L⁻¹) caused decubitus in 50% of fishes but without any changes on VF (P=0.07), while propofol (0.15 mL L⁻¹) causes decubitus in 75% of fishes and had a significant difference between basal and VF in decubitus stage (P =0.0003). At these concentrations, time for decubitus was longer than other higher concentrations (P< 0.001). The two higher concentrations tested (benzocaine: 13 and 26 mL L⁻¹; propofol: 0.6 and 1.2 mL L⁻¹) cause decubitus in 100% of fish and caused complete loss of VF for one minute during this stage (Figure 3).

Initial VF between control group, benzocaine and propofol did not differ from each other (p=0.99). Although there was no difference in latency to anesthesia induction (P =0.92), time to decubitus and time to recovery were longer in the propofol group (P =0.0001). Propofol also reduced VF to lower levels when compared to benzocaine (P =0.0003) (Figure 4). Fish returned to basal levels of VF only in the benzocaine group.

Fish submitted to propofol took a longer time to feed (P =0.00002) (36.67 ± 9.87 min after food was offered). Benzocaine (9.27 ± 3.32 min) and control group (5.9 ± 4.53 min) had no difference in latency to feed (P =0.08).

Discussion

Anesthesia induction by immersion in propofol was evaluated for the first timein Nile tilapia. Propofol, used in the form of a water bath, produced safe anesthesia for Nile tilapia. Propofol is an effective alternative fish anesthetic that could be used in situations that require fast and longer action, such as in fish management in aquaculture farms and health procedures such as surgeries and vaccination. On the other hand, benzocaine can be used as a less powerful anesthesia with fast recovery.

Time to induce decubitus was also different between anesthetics and ventilatory frequency (VF). Benzocaine had maximum effect on VF only at 6.5 mL L⁻¹ or higher, with significant reduction at the end of decubitus stage. Similarly, propofol had significant reduction in VF at doses of 0.3 mL L⁻¹ or higher. According to Marking and Meyer (Marking and Meyer 1985), latency close to 3 minutes to enter decubitus stages is acceptable and it is a threshold to consider an anesthetic as efficient in fish. Induction should occur quickly in fish, preferably within 3–5 min, to minimize hyperactivity reactions or stress (Ross et al.
This criterion was achieved by benzocaine and propofol on 6.5 mL L\(^{-1}\) and 0.3mL L\(^{-1}\), respectively. Considering our results, these concentrations of benzocaine and propofol must be the minimal dose to anesthetize juveniles of Nile tilapia.

Regarding the effects of the anesthetics, there was no difference in VF between groups before submitting fish to anesthetics. Therefore, differences after exposure are due to anesthetic process. However, propofol decreased the rate of gill ventilation with time of exposure, and thus longer exposure may be dangerous, probably leading to severe hypoxia. According to Ross (Ross 2001) anaesthetics can produce progressively deeper anesthesia (even during recovery in anaesthetic free water) due to anaesthetic concentration increase in the brain and muscle despite its blood equilibration.

Fig. 2 Comparison of time to induce decubitus and recovery from anesthesia after anesthetic application in four different dosages. Letters means significant differences and NS means not significant (p ≤ 0.05; ANOVA).

Fig. 3 Evolution of ventilatory frequency of Nile tilapia over anesthesia (benzocaine and propofol) stages in different doses. Letters means significant differences at the same dosage between VF levels of anesthesia stages (uppercases for benzocaine and lowercases for propofol; P≤ 0.05; ANOVA). Basal: initial VF; decubitus: VF on anesthesia induction; Change: VF during four minutes of decubitus; Return: VF of recovery of normal posture and Rest: VF four minutes after return.
In addition, the average time to induce decubitus was the same between benzocaine and propofol. However, recovery time was longer in fish bathed in propofol than those submitted to benzocaine, meaning that the former is more potent than the latter. Thus, the choice between benzocaine and propofol will depend on usage. For example, for fish industry, it would require a massive anesthesia and longer durability of anesthesia, ensuring stunning during slaughter process and some scientific protocols that demand longer time of stunning (i.e. invasive procedures). In these cases, Propofol would be indicated instead of benzocaine. On the other hand, some scientific methods and industry procedures (i.e. tagging fishes, vaccine application) would require agility on stunning and recovery process, when benzocaine would fit better.

Despite these observed properties of benzocaine and propofol, the usage for fish industry to produce meal needs careful attention, since these anesthetics can deteriorate the quality of product. Benzocaine was already assessed by United States Foods and Drug Administration (FDA) that have established a maximum concentration of 50 ppb on fish muscles, specifically on Atlantic salmon and rainbow trout. The rate of elimination of propofol from trout blood was similar to those reported for benzocaine. The half-life time for propofol was 1.5 h and 1.1 h for water temperatures of 12 ºC and 17 ºC, respectively (Gomułka et al. 2015). Stehly (1998) found half-life times for benzocaine as 1.0 h, 0.6 h and 0.7 h for 6 ºC, 12 ºC and 18 ºC, respectively. According to Guénette et al. 2007, the plasma half-life time of eugenol was 12.1 h and the authors suggest its potential tendency to accumulate in trout tissues. As temperature affects propofol elimination from trout blood, a longer time of recovery should be expected at lower water temperatures. A study on propofol elimination from Nile tilapia blood and tissues in low temperatures is needed.

Latency to feed follows the same pattern. Food ingestion at recovery case is associated with the end of anesthetic effects, when animals return to their normal activities. Food ingestion returned faster when fish were anesthetized with benzocaine than propofol. Latency to food ingestion can also be related to stress in fish. Catabolic actions in metabolic processes are considered the main secondary responses to stress, and affects food intake, growth and reproduction (tertiary stress response) (Pottinger 2008).

This strong and deep action of propofol demonstrated in Nile tilapia seems to be due to its mechanism. Propofol acts on CNS, modulating GABAergic inhibitory activity (Sonner et al. 2003). While benzocaine acts blocking Na+ entrance in neurons, blocking neurotransmission (Elliott et al. 1987) and inhibiting and propagation of action potentials on peripheral systems (Frazier and Narahashi 1975; Neumcke et al. 1981; Sneddon 2012), mainly muscles as little effect is observed on nerve membranes (Matthews and Varga 2012). This peripheral way of action can make this anesthetic less powerful and superficial, when compared to propofol.

Considering these effects and clinical relevance, propofol has a more intense and longer effect when compared to benzocaine. Thus, in situations where light anesthesia with faster recovery is required,
benzocaine treatment would be recommended. However, in situations where a profound anesthesia is desirable, requiring prolonged and deep effects, propofol would be recommended, especially for procedures such as translocation and surgeries. This study only investigated acute exposure, thus future studies should focus on chronic effects of these anesthetics and the safety of propofol usage for fish food production.

**Conclusion**

We found propofol anesthesia in Nile tilapia effective and safe; however, during propofol anesthesia the respiratory rate decreases gradually, and therefore a specific detailed anesthesia protocol should be developed.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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