Ethics and humane practices of bleeding and euthanasia for experimental marine fishes in fish nutrition research

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Scientific research in fish consisting haematological, biochemical, bacteriological, parasitological, toxicological, immunological, somatic measurements, tagging, digital photography for morphometrics and reproductive investigations requires anaesthetization. An overdose of anaesthetic is also an effective method to euthanize fish humanely whenever necessary. The recommendations of universal ethical committee mentions that the fish should be relieved of pain (analgesia) through block of pain perception with or without the retention of other sensory abilities while collecting the blood or euthanasia.

Anaesthetization

Sedation is a preliminary state of anaesthetization and on continuation drowsiness is induced, with dulled sensory perception and with some analgesic effect (insensitivity to pain), but no gross loss of sensory perception or equilibrium. In fish, the light and deeper sedation has been identified by responsive to stimuli through reduced motion and ventilation. The progressive stages of sedation is called anaesthesia with three obvious phases such as induction, maintenance and recovery. The light anaesthetic response can be defined as partial loss of equilibrium and good analgesic effect and the deeper anaesthesia is indicated through total loss of muscle tone, total loss of equilibrium and almost nil ventilation. The last stage of anaesthesia is called as medullary collapse in which ventilation ceases, cardiac arrest and eventual death that occurs through overdose either through prolonged exposure of higher anaesthesia concentration (McFarland, 1959).

For euthanasia, fish may be given appropriate dose and duration of deeper anaesthesia to give complete pain relief. Therefore, for each fish the anaesthesia should be selected appropriately and dose should be optimized to get better effect on induction, maintenance and recovery of anaesthesia. The induction phase should provide quick ataxia (loss of equilibrium) and loss of the righting reflex and the maintenance phase involves extending the achieved state of anaesthetic effect in a stable manner without detriment to the health of the fish. The recovery phase involves withdrawal of the anaesthetic agent and return to a normal state in time duration of few seconds to a few minutes, without altered behaviour or side-effects. Among the several anaesthetic agents, tricaine methanesulfonate (MS222) and clove oil are very safe and does not have many side effects in marine fishes.

Blood collection in marine fishes

Several methods such as caudal venous puncture, dorsal aorta puncture, tail ablation, decapitation, severance of caudal vein and heart puncture (MUAWC, 2008) are available. The blood collection technique is chosen based on several factors such as the size, health status, the quantity of blood required and the fate of the fish (sacrificed or rescued) in a study. Most of these methods are destructive as fish may be killed during blood collection. While collecting blood, if the fish becomes too much stressed it can compromise the parameters studied. In caudal venous puncture method, the fish can recover to its original health within a few days post blood collection. Therefore, marine fishes such as snubnose pompano, Asian sea bass, orange spotted grouper, cobia and mangrove red snapper are subjected to standardized caudal venous puncture method for fish nutritional research. In general,

the volume of blood in most of the fish is about 6% of its body weight and hence, for any serial blood collection study, the blood should not be withdrawn beyond 1/6th of the total volume available in the fish.

In ICAR-CMFRI the anaesthetization protocol was standardized using clove oil and MS222 for snubnose pompano and orange spotted grouper. For blood collection, the is transferred carefully and with least stress into the vessel with anaesthetic agent. The fish is allowed to swim until with ataxia, it loses its equilibrium and gets fully anaesthetized. When it completely loses its equilibrium, it should be taken out and kept in soft wet cotton cloth to prevent mucous sloughing during sampling. It should be ensured that the fish does not show any movement (opercular movement and muscle tone) and therefore does not feel pain during the blood collection process.

The needle and syringe should be selected according to the size of the fish (Table 1). The needle should be sterile and changed for every fish to avert blood clotting while collecting blood. For small size fish, 26 G needle fixed in 1 mL tuberculin syringe is ideal and as the size of the fish increases, the needle and syringe size should be selected. The needle should be rinsed with anticoagulant (2.75% EDTA solution or heparin) to avert blood coagulation during the course of blood collection. The needle is inserted beneath the scales in the caudal peduncle area, just below the lateral line at about 45° angle to the lateral surface in a cranial or ventral direction, until it touches the vertebral column. This can be felt by hard impenetrable surface and thereafter, the syringe is withdrawn slightly, approximately 1-2 mm, so that the blood vessel beneath the vertebral column can be pierced and sampled. After blood collection, the fish is released into a recovery tank with freshly aerated aesthesia-free water and after the anaesthetic effects are withdrawn the fish starts to swim freely.

Blood collection from snubnose pompano by caudal venous puncture

Plasma and serum collection and storage

For the preparation of plasma, the collected blood is transferred carefully into anticoagulant coated vacutainer collection vials [EDTA-treated (lavender tops); citrate-treated (light blue tops); heparinized tubes (green tops) or sodium fluoride tainted (grey top)] rapidly by removing the needle to prevent haemolysis. The blood is mixed gently with anticoagulant in the container and should be used for further analysis within 3 hours at room temperature. It can be stored for 24 hours at refrigerated temperature (4 °C). The plasma is collected by removing blood corpuscles through centrifugation at 5000 rpm for 10 minutes in a refrigerated centrifuge. The supernatant plasma can be preserved at 2–8°C for few days and at –20°C for prolonged period, without much freeze-thaw cycles. For serum collection, the blood should be released into the anticoagulant free container (red top) and kept in slanting position. The blood is allowed to clot by leaving it undisturbed at room temperature for 2 hours for better separation of serum. The clot portion is removed by centrifugation at 5000 rpm for 10 minutes in a refrigerated centrifuge. The resulting supernatant serum is transferred into a clean polypropylene tube. The

Table 1. Dimensions of needle and syringe in relation to fish size

Fish size	Needle Gauge	Outer diameter (mm)	Inner diameter (mm)	Syringe
<25 g	26 G	0.46	0.26	1 mL
25-100 g	24 G	0.57	0.31	1 mL / 2 mL
100-500 g	22 G	0.72	0.41	2 mL
0.5-2 kg	20 G	0.91	0.60	5 mL

serum samples can be stored for 7-10 days at 2–8°C. If the serum is not analyzed immediately, it should be apportioned into 0.5 mL aliquots and stored at –20 °C until analysis not exceeding 3 months. It is advisable to avoid frequent freeze-thaw cycles because this is detrimental to many serum components.

Euthanasia is generally practiced to collect the tissue samples from the experimental fishes by sacrificing them after completion of experiments to assess the enzymological, biochemical, immunological assays and reproductive performances. In such situation the fish should be killed humanely, without giving any pain, that can be achieved through overdosed anaesthetization (Table 2).

Table 2. Dosage of anaesthetics commonly used for anaesthesia and euthanasia in fish

Anaesthetic	Dosage*	
	For anaesthesia	For euthanasia
MS-222	75-150 mg L^{-1}	1 g L
Clove oil	0.2-0.5 mL L^{-1}	> 1.5 mL L^{-1}
Benzocaine	100 mg L^{-1}	-
Etomidate	1-4 mg L^{-1}	-

*Dosage may vary with species, age and size of fish

Reference:

McFarland, W.N., 1959. *Publ. Inst. Mar. Sci.*, 6: 22–55.

MUAWC, 2008. Monash University Animal Welfare Committee, pp. 1-10.