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Review
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BLOOD SAMPLING TECHNIQUES AND PREPARING FOR ANALYSIS IN RAINBOW TROUT (ONCORHYNCHUS MYKISS)

Utku DURAN[1]*, Sena ÇENESİZ[2], Büşra ŞAHİN[2]

[1]*Zonguldak Bülent Ecevit University, Çaycuma Vocational School of Food and Agriculture, Laborant and Veterinary Health Program 67900, Zonguldak, Türkiye*
[2]*Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Biochemistry, 55139, Samsun, Türkiye*

Abstract: In aquaculture, biochemical and hematological analyzes are frequently performed for scientific research, health screening and diagnosis of diseases. Biochemical and hematological parameters in fish as in other vertebrates varies nutrition, water quality, pathogens and various environmental factors that can create stress. Caudal vascular blood collection is a non-invasive method that is widely used to investigate fish health, biochemistry, and physiology. As a result of this method being performed under the influence of a properly selected anesthetic agent, animal welfare is affected the least, thus preventing a serious change in biochemical parameters with minimum stress. In this review, sampling collection suitable for the analysis to be performed and choosing the right anesthetic agent are presented.

Keywords: Rainbow trout, Blood sampling, Biochemistry, Hematology

***Corresponding author:** Zonguldak Bülent Ecevit University, Çaycuma Vocational School of Food and Agriculture, Laborant and Veterinary Health Program 67900, Zonguldak, Türkiye
E mail: utku.duran@beun.edu.tr (U. DURAN)
Utku DURAN https://orcid.org/0000-0002-0002-8893
Sena ÇENESİZ https://orcid.org/0000-0002-3544-503X
Büşra ŞAHİN https://orcid.org/0000-0002-5245-478X

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1. Introduction

Hematological and biochemical analyzes in fish are widely used methods to evaluate the physiological state and health of fish. Some of the hematological parameters are very sensitive to the change of environmental factors. All preanalytical and analytical factors can affect the results. Therefore, experience and care is required to obtain reliable hematological data (Witeska et al., 2022). Caudal puncture is a commonly used technique for blood collection in live fish. With adequate practice and improvement of procedures, it is possible to take a blood sample from the caudal vascular system of a fish, especially in large fish, without the use of even anesthetics (Lawrence et al., 2020). Various methods are used to induce sedation in fish to prepare them for sampling and to facilitate caudal puncture. For this purpose, pharmaceuticals (MS-222, Clove oil, 2-phenoxyethanol Etc.) (Martins et al., 2018) and physical influencers (electric current) (Reid et al. 2019), lowering water temperature and CO_2 (Erikson, 2008) can be applied.

The aim of this review is to compile the factors that may affect the results and to present information for researchers who will work in this field, taking into account the studies in which the blood collection process in fish is performed.

2. Various Factors That Can Affect Blood Biochemistry

Reference ranges for hematological and serum biochemical parameters have been extensively studied in humans and other mammals because of their critical importance in the diagnosis of various pathophysiological conditions (Nabi et al., 2022). Studies on fish biochemistry in veterinary medicine are few compared to human medicine. For this reason, studies in the human field have been expanded in the veterinary field (Reshma et al., 2020). Many authors have revealed the difficulty of determining normal reference values related to hematology and serum biochemistry in rainbow trout (*Oncorhynchus mykiss*) (Wedemeyer and Chatterton, 1970; McCarthy et al., 1973; Hille, 1982; Fazio, 2019). Biochemical blood parameters in fish change due to biological and environmental factors. Biological factors include fish species, population, reproduction and sex. Environmental factors, on the other hand, are the changes in the physicochemical properties of water depending on climate or eutrophication and stress. The situation does not change in rainbow trout. In order to correctly interpret the blood values observed in fish, the factors affecting them should be known and taken into consideration.

Except for recirculated systems, the changes in the water should be carefully monitored before taking samples from the fish. Environmental pollution, biological and chemical wastes, and the pesticides (Sertçelik et al., 2018) used in the fight against agricultural pests may directly affect non-target organisms. The negative effects that may occur in any organism of the ecosystem may also affect the rest of the ecosystem through food chain or interchangeable interaction (Tunçsoy et al., 2021). The pesticide contamination following the use of organophosphorus pesticides even in sublethal doses may occur as a result of reaching the aquatic ecosystem and change biochemical parameters in the bodies of fish species that constitute non-target teleost populations (Kaya et al., 2021). In a study conducted by Deveci et al. (2016), it was revealed that the frequently used pesticide Chlorprifos-Ethyl caused oxidative stress in fish due to an increase in reactive oxygen species. Successful blood collection and obtaining reliable hematological analysis results are highly dependent on the fish itself, environmental conditions and especially the stress level at blood sampling. Some of the stress factors that can lead to biochemical changes are the preparation of fish during blood collection, transportation of the cage from one place to another, exposure to air during transportation, hypoxia, and an inexperienced person dealing with blood collection (Pollock et al., 2007; Fagundes and Urbinati, 2008; Aguirre-Guzman et al., 2016; Witeska et al., 2022).

Stress-induced haematological changes depend on the type, size, and time of action of the stressor (Ruis and Bayne 1997). Stress can cause changes on erythrocytes and biochemical parameters (eg glucose, catecholamine, enzyme activity, and cortisol etc.) (Sugeçti, 2021a; Sugeçti, 2021b; Witeska et al., 2022). There is an increase in MCV and Ht values due to stress. An increase in the number of RBCs has also been reported depending on the duration of the stressor (Dobšíková et al., 2009; Aguirre-Guzman et al., 2016; Fazio, 2019). There is also a change in the number of leukocytes depending on the duration of the stressor. While there is an increase in the number of WBCs due to short-term stress, chronic stress causes leukopenia (Tort, 2011). Stress also causes changes in the values of biochemical parameters. There is an increase in glucose, lactate and cortisol levels, especially with the effect of adrenaline (Martínez-Porchas et al., 2009; Pankhurst, 2011; Refaey and Li, 2018).

In a study conducted by Braun et al. in 2006, it was revealed that there were changes in serum catalase activity in fish exposed to hypoxia (Braun et al., 2006). In addition, in a study conducted by Keleştemur et al.in 2010, reported that lipid peroxidation in blood and tissues started with the increase in serum MDA level due to stress in rainbow trout fry during transplantation (Tuna Keleştemur et al., 2010).

3. Correct Anesthetic Selection and Importance of Dose

Fish can be physically restrained without anesthesia. However, this can cause stress and biochemical changes. It is also not suitable for animal welfare (Barton, 2002; Acerete et al., 2004;Ramsay et al., 2009). General anesthesia is defined as a temporary loss of sensation through depression of the central nervous system. Anesthetics primarily serve to immobilize a fish. Therefore, these agents can provide conditions for rapid blood collection from the animal without physical restraint and to minimize stress-related effects (Lawrence et al., 2020).

Since non-lethal blood or sampling procedures are minor surgical procedures, mild anesthesia or sedation of the fish would be the right choice. Also, using a light dose of anesthetic will speed up the recovery of the fish (Javaher et al., 2012; Trushenski and Bowker, 2012; Smith et al., 2017). In addition to choosing an appropriate anesthesia protocol, the correct dose and correct anesthetic agent selection is very important. Otherwise, fish welfare may deteriorate or data may change. In addition, death may occur in fish exposed to too much anesthetic. Anesthetics commonly used for this purpose, MS-222 and 2-phenoxyethanol, Metomidate and Ketamine can be used singly or in combination (Martins et al., 2018).

There are also some disadvantages of using anesthesia during blood collection from live fish. The most important of these is the slow induction and long awakening time, which causes the fish to be under anesthesia more than necessary due to the short nonlethal blood collection time (Hikasa et al., 1986; Mylonas et al., 2005; Neiffer and Stamper, 2009).

Finally, after the use of anesthetic substances, legal washout periods should be waited for the consumption of fish. Especially in aquaculture, consumption of fish must be prevented after sampling. The wash-out time varies for the anesthetic to be used. For example, for fish using MS-222, the US Food and Drug Administration (FDA) recommends a 21-day washout period for the chemical to leave the tissues. In addition, the FDA recommended dose for sedation with MS-222 is 15 - 50 mg/L, for anesthesia 50 - 200 mg/L (FDA, 2020).

4. Suitable Blood Collection Regions for Rainbow Trout

4.1. Cardiac Puncture

The cardiovascular system in fish is relatively simple compared to other vertebrates. It consists of two main components, the heart and the peripheral vascular system. Due to the absence of the diaphragm found in mammals in fish, the body cavity is in one piece. The heart is located cranially in the main body cavity, inside the pericardium. The heart is a four-chambered structure consisting of *sinus venosus*, *atrium*, *ventricle*, and *bulbus arteriosis*. It also has a one-way flow of deoxygenated blood. Deoxygenated blood is collected posterior to the hepatic veins to enter the *sinus venosus* via a two-channel duct system from the anterior of the fish. *Sinus venosus* opens into the atrium with a valve. The *atrium* has thick muscular walls and pumps deoxygenated blood into the *ventricle* (Eissa, 2016). The *ventricle* is relatively large enough to receive blood. In adult rainbow trout, the heart can reach 1.5 – 2 cm in size. Considering the contraction of the heart, it is possible for the cannula to come out of the heart. Therefore, blood collection by cardiac puncture is not a practical method (Duman et al., 2019).

4.2. Dorsal Aortic Puncture

By means of a hypodermic needle tip, repeated blood collection from the dorsal aorta at the roof of the oral region is possible. For this procedure, the fish must be anesthetized with a suitable anesthetic. The needle tip is inserted into the dorsal aorta at an angle of approximately 45 °C, just behind the 3rd and 4th gill arches. Eight vessels from the gills join the dorsal aorta just before this point. This technique is suitable for blood pressure studies as well as facilitating sampling for hematological analysis (Schiffman, 1959). For rainbow trout between 60 and 1200 grams, a 22 Gauge needle tip can be used to draw blood with this technique. However, since the mouth of the fish must be open for blood collection from the dorsal aorta, it will not provide a suitable working area for small fish. This process can be used to obtain the appropriate amount of blood in adult fish.

4.3. Caudal Vein Puncture

Depending on the size of the fish, 21 and 23 Gauge needle tips can be used for Caudal Vein Puncture. Generally, the caudal vein is located in the haemal arch dorsal to the vertebral arm. As a result, reaching the tip of the syringe into the vertebral column is the primary cue for finding the caudal vein (Lawrence et al., 2020). For this process, it is recommended to enter the fish at an angle of 45 °C just behind the anal fin and proceed up to the vertebral column. If the blood does not start coming from this point, the syringe is pulled back a little and the vein is reached. However, due to the asymmetrical cut of the needle tip, it is also possible that the blood does not come out despite being in the vein. In order to prevent this, turning the needle tip a little can help the blood to enter the needle tip. Generally caudal puncture is suitable for haemal posterior reach for most fish species (Jeffries et al., 2011; Lawrence et al., 2018). Another advantage of caudal puncture is that it is done behind the anal fin. So, it does not involve the risk of damaging vital organs. For this reason, caudal vein puncture is the most suitable procedure for repeated blood draws (Reid et al., 2019) (Figure 1).

5. Blood Draw Amount

In trout and other teleost fish, the blood volume is about 3% of their body weight. For nonlethal blood collection techniques, large volumes of blood collection in a short time can cause adverse physiological consequences in fish such as hemodilution, cardiovascular changes, lethargy and temporary loss of balance due to hemorrhagic shock, including neuroendocrine responses (Schiffman, 1959; Duff and Olson, 1989). There is no widely accepted rule for how much blood can be drawn from live fish. However, the Canadian Department of Fisheries and Oceans considers it appropriate to take up to 0.1% blood (Canada Department of Fisheries and Oceans, 2004).

6. Equipment Selection

Various sizes and lengths of needles, injectors (vacutainer or manual) and tubes are needed for blood collection. Needle size and length vary depending on the size of the fish, but vary between 20 – 30 Gauge. The tube should be selected in accordance with the analysis to be made. For example, in tubes to be used for the analysis of serum biochemical parameters, silica gel tubes should be preferred because it facilitates the separation of serum in centrifuge (Duman et al., 2019). Vacutainer tubes are classified according to cap colors and analyzes to be used. Accordingly, for serum collection, bacteriological and parasitological analysis, the use of a chemical-free red capped tube, for clinical biochemistry parameters and plasma collection a green cap (with lithium or sodium heparin), for hematological examination a purple cap (with EDTA), and for coagulation studies light blue cap (sodium citrate) it is appropriate to use tubes. Pediatric tubes or manual syringes should be used when collecting blood from small fish, as the use of a very large tube or syringe will cause severe hemorrhagic shock in small fish (Canada Department of Fisheries and Oceans, 2004; Duman et al., 2019; Duran, 2019) (Table 1).

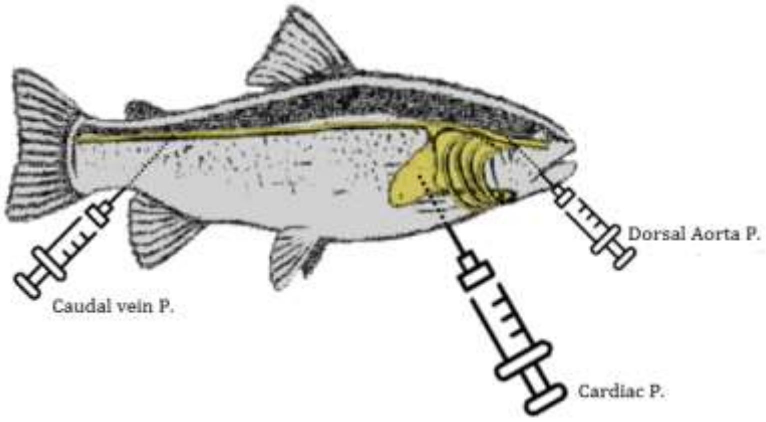

Figure 1. Schematic representation of different body parts for blood collection from fish.

Table 1. Blood collection equipment to be selected according to the size of the fish.

Size (g – cm)		Needle (Gauge)	Syringe (Capacity)	Methods
Larva		Hematocrit glass capillary tube	50 – 75 uL	Tail cuting
Fry (< 5 cm, < 7 g)		30 G	1 cc	Caudal vein P., Cardiac P.
Juvenil (< 250 g)		29 G, 25 G,	1 cc	Caudal vein P., Cardiac P.
Adult (300 g – 3000 g)		23 G, 22 G, 19G	2.5 cc, 5 cc	Caudal vein P., Dorsal aorta P.

7. Equipment Selection

Blood taken into sterile tubes should be brought to the laboratory by paying attention to the cold chain before starting the study. For serum isolation, blood should be kept at +4 °C for at least 1-2 hours in order to allow blood to clot. Coagulation is not possible since 10% EDTA is sampled for plasma isolation (Worldfish/CGIAR, 2019).

It is recommended that the blood taken into dry and clean tubes with yellow or red caps for serum isolation and EDTA tubes for plasma separation should be centrifuged at +4 °C for 7000 G x 15 minutes. For serum and plasma isolation, it is recommended to take at least 200 µL of blood from each fish (Ideally > 500 µL) (Worldfish/CGIAR, 2019). Blood volume scale with body size, that typically 3-4% of body mass in teleost fishes (Olson, 1992). This ratio shouldn't be passed for nonlethal blood drawing in rainbow trout.

Plasma/Serum preparation steps:

1. Draw the appropriate amount of blood for the size of the fish within 3 minutes of being caught.
2. Remove needle and dispense blood into to centrifuge tube.
3. Transport to lab on ice or cool packs
4. Allow blood to coagulate for a minimum of 2 – 4 hours at room temperature for serum isolation.
5. Centrifuge tubes at 7000 x G for 15 minutes.
6. Collect serum (upper clear supernatant layer).
7. Transfer serum to new sterile tube (store at –20 °C or –80 °C for future analysis)

8. Conclusion

The circulatory system provides important data for monitoring the health and physiology of fish. Taking blood samples from live fish with appropriate equipment is a common practice for researchers. The correct selection of the techniques and equipment used is very important for the reliability of biochemical and hematological analyzes. In addition, it should not be forgotten that even the easiest of these processes creates stress on the fish. In terms of animal welfare and reliability of analysis, studies have shown that the most appropriate method for nonlethal blood sampling, including small fish, is caudal vein puncture, and in large fish, both dorsal aorta and caudal vein puncture. In this review, blood collection techniques and correct equipment selection for rainbow trout, which is the most cultured in the world, are presented.

Author Contributions

The percentage of the author(s) contributions is present below. All authors reviewed and approved final version of the manuscript.

	U.D.	S.Ç.	B.Ş.
C	40	30	30
D	25	50	25
S	40	30	30
L	40	30	30
W	25	25	50
CR	50	25	25
SR	50	25	25

C=Concept, D= design, S= supervision, L= literature search, W= writing, CR= critical review, SR= submission and revision.

Conflict of Interest

The authors declare that there is no conflict of interest.

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