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Comparative Physiological Aspects of Plasma Hemostasis of some Commercial Fish Species

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ABSTRACT

The hemostasis system is designed to ensure the integrity of the internal environment of the body and bleeding control and maintain the liquid state of blood in the vasculature. Clinicodiagnostic characterization of clotting in different fish species contains incomplete and scarce information in modern biological science. The data obtained revealed some issues of functioning of the ancient mechanism of blood clotting in these animals in both evolutionary and veterinary aspects. Thus, a comparative study was conducted on the functional state of plasma hemostasis in some commercial fish; namely, cartilaginous ganoids sturgeon (*Acipenser baerii)*, hybrid of sterlet (*Acipenser ruthenus*), starred sturgeon (*Acipenser stellatus*), bony – common carp (*Cyprinus carpio*) and the tilapia, *Oreochromis niloticus*. The following coagulogram parameter changes were addressed: thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (APTT), the concentration of fibrinogen, and soluble fibrin monomer complexes (SFMC). It is assumed that the functioning of the plasma hemostasis system has species specificity regardless of stress resistance, especially in commercial fish at different evolutionary stages. Hence, species-specific characteristics of fish clotting were determined. Activation by the common and extrinsic pathways was several times faster in cartilaginous ganoids (reduced TT by 17.8-26 times, PT by 1.9-2.6 times, reduced fibrinogen concentration by 4.8-6.11 times) than in both the species of bony fish. The hemostasis, with activation of the intrinsic pathway (APTT), was faster in the hybrid and the tilapia by 20.6-16.8 times compared to that in sturgeon, and by 2.8 and 2.2 times compared to carp. The SFMC content in all fish was (1.3-4.3 times) higher than that noted in dogs and 2.5-8.3 times higher than the SFMC content in humans. But, it was lower (1.0-2.4 times) compared to that recorded in cattle. The highest/lowest values of SFMCs were detected in carp and the hybrid of *A. ruthenus* and *A. stellatus*, respectively.

INTRODUCTION

The clotting (blood coagulation) system has won a considerable interest with respect to the veterinary, medical, and evolutionary points of view. The hemostasis system is designed to ensure the integrity of the body's internal environment and stop bleeding in case of any damage in the vascular wall, preserving its permeability and resistance, and maintaining the liquid state of blood in the vascular channel. The

coagulation mechanism is explained by A. Schmidt's enzyme theory and blood coagulation is based on three interrelated phases: thromboplastin formation, thrombin formation, and fibrin formation **(Kudryashov, 1975; Amineva & Yarzombek, 1984)**.

The first phase of blood coagulation (prothrombinase formation) is characterized by APTT. It describes the intrinsic pathway of coagulation activation, prothrombin formation, and is a multistep process that results in the accumulation of a complex of factors in the blood that can convert prothrombin into thrombin. PT indicates the activity of the extrinsic clotting pathway. PT characterizes the first (prothrombinase formation) and second (thrombin formation) phases of plasma hemostasis and reflects the activity of the prothrombin complex. The third phase of blood coagulation (fibrin formation) is assessed by using fibrinogen and TT values. TT is a screening test of the last phase of blood clotting, reflecting the rate of fibrinogen to fibrin conversion **(Bonar *et al.*, 2017)**.

It is known that in some animals, the hemostasis system has reached great perfection. The main features of this process are studied in detail in higher animals and humans, but are much less investigated in lower animals **(Botyazhova, 2000)**. Thus, there is a natural interest in the principles of the structural and functional organization of hemostasis in different animal species, including fish with the peculiarities of their habitat and evolutionary stage.

The clotting system appeared before the descent of tetrapods and bony fishes about 430 million years ago **(Davidson *et al.*, 2003)**. The hematological triad of roundworms, cartilaginous fishes, and cartilaginous ganoids formed the main evolutionary path of the blood system by natural selection. The intrinsic, extrinsic, and common pathways of the clotting system of marine and freshwater bony fishes have been described in many studies **(Doolittle & Surgenor, 1962; Lewis, 1996; Pavlidis *et al.*, 1999; Tavares-Dias & Oliveira, 2009)**, including theoretical and experimental aspects. In addition, the genes of multiple factors comprising the hemostatic response cascade have been characterized, and the ways of this essential function formation in phylogeny have been proposed **(Rowley *et al.*, 1997; Davidson *et al.*, 2003; Jiang & Doolittle, 2003; Jagadeeswaran *et al.*, 2005; Doolittle, 2009, 2012)**.

Rapid blood clotting is of great importance for fish life, especially benthic fish. Studies carried out on bony fishes indicate that the coagulation process is fundamentally similar to other vertebrates, particularly mammals, with the only difference being that it is adapted to lower temperatures. The main thrombogenic protein components have been found in fish, including thrombotropin, prothrombokinase and thrombokinase, prothrombin, thrombin and fibrinogen **(Doolittle & Surgenor, 1962; Tavares-Dias & Oliveira, 2009; Zhichkina *et al.*, 2017)**. The results of the above- mentioned genetic and routine laboratory screening tests used for human blood **(Van Vliet *et al.*, 1985; Kawatsu, 1986; Smit & Schoonbee, 1988)** show that fish blood clotting factors are similar to those in mammalian or human blood, and the coagulation cascade also involves three classical processes (phases).

Enzymes involved in the blood clotting of fish can work in a wider range of temperatures than in warm-blooded species **(Botyazhova, 2000)**. In fish (loach, perch, sterlet, sturgeon, carp, gudgeon), blood clotting is almost instantaneous, i.e., within 10-12 seconds, whereas in mammals and birds – within 2-12 minutes **(Zhichkina *et al.*, 2017)**. Skin mucus, which is believed to contain a large amount of thrombokinase, serves as a

process accelerator **(Kudryashov *et al.*, 1958; Golovina, 1996; Botyazhova, 2000; Zhichkina *et al.*, 2017)**.

The main differences between blood clotting in fish and that in mammals lie in the predominance of internal conversion of prothrombin to thrombin in the latter, while the extrinsic pathway is probably similar. Platelets in fish play a central role in the internal conversion of prothrombin to thrombin and are responsible for clot retraction, although the nature of platelet factors promoting clotting is unknown **(Doolittle &Surgenor, 1962)**.

As for bony fish of fishery importance, some data on secondary (plasmic) hemostasis in bony fish cover a small number of freshwater species, such as the catfish *Ameiurus nebuiosus* (**Langdell *et al.*, 1965**), carp *Cyprinus carpio* (**Fujikata, 1985; Fujikata & Ikeda, 1985a, 1985b; Kawatsu, 1986; Kawatsu & Kondo, 1989; Kawatsu *et al.*, 1991; Jung & Kawatsu, 1994)**, rainbow trout *Onchorynchus mykiss* **(Ruis & Bayne, 1997)**, and tilapia *Oreochromis mossambicus* **(Smiley *et al.*, 2001)**. The work of J. H. Lewis studied the coagulation cascade of vertebrates, including cartilaginous fish, bony fish, and cartilaginous ganoids. This work quantitatively characterized the hemostasis functioning of such valuable fish species like sturgeon, arctic trout, flounder, sea bass, mullet and others **(Lewis, 1996)**. These studies revealed differences in the clotting time and the content of certain clotting factors in different groups of fish and emphasized the need to use validated and uniform procedures (e.g., nature of thromboplastin used, type of laboratory dishes) in hemostasis studies of these hydrobionts. **Ivanov (2021)** suggested that interspecies' differences in blood clotting in fish may well be the result of differences in the resistance of these fish to stress. It is assumed that the functioning of the plasma hemostasis system has species specificity regardless of stress resistance, especially in commercial fish belonging to different classes.

An important point emphasizing the practical component of such studies is spontaneous thrombus formation described in milkfish *Chanos chanos*, skipjack *Katsuwonus pelamis*, yellowfin tuna *Thunnusalbacares*, and mullet *Mugil cephalus* **(Smith, 1980)**. Additiionally, it is not uncommon for fish in aquaculture to die unexpectedly a few days after traumatic manipulation, such as sorting. This probably occurs due to trauma capable of causing spontaneous thrombosis **(Smith, 1980)**.

Based on the above data, the goal of the current study was to present a comprehensive assessment of the plasma component of the hemostasis system with respect to some common species of commercial fish. To hit well this target, researchers had to solve the following tasks:

- Investigating the functional state of the plasma component of hemostasis in commercial species of cartilaginous ganoids and bony fishes;
- Comparing the pathways of coagulation activation in fish of different classes.

MATERIALS AND METHODS

Materials

The work was performed at the Aquaculture Development Center "AquaBioCenter" in the period extending from 2015-2020. Under aquarium conditions, studies were conducted on important world fishery species of bony fishes (*Osteichthyes*) belonging to two different classes. Animal units of the bony fish class (*Teleostei*) - common carp *Cyprinus carpio* L. and the Nile tilapia *Oreochromis niloticus* L. were used in the study. Animal units of Siberian sturgeon *Acipenser baerii* B. and hybrid of sterlet *Acipenser ruthenus* L. and starred sturgeon *Acipenser stellatus* P. were taken as cartilaginous ganoids (*Chondrostei*). The study of *Acipenseridae* is of particular interest because they are members of an ancient group that is closer to the main path of vertebrate evolution than bony fishes **(Long, 2011)**. All fish species had a large mass and were suitable for hemostasiological studies involving the taking of large volumes of biomaterial. Fish were farmed commercially in the fish company, Diana LLC (Vologda Region) and AquaBioCenter. The fish were kept in aerated aquariums; cold-loving at 16-18℃, heat-loving at 28-30℃. Acclimatization period before the study was 48 hours. Before blood sampling, fish were anesthetized by adding clove oil in water at a dose of 0.033 ml/l **(Hamackova *et al.*, 2006)** with subsequent exposure for 15 minutes. Blood sampling was performed into glass tubes by puncturing the caudal hemal canal with 3.8% sodium citrate using a plastic syringe. The study object was platelet-poor plasma (PPP) obtained by blood centrifugation for 15 min at 3000 xg.

Methods

The plasma coagulation hemostasis parameters were determined on a «Thrombostat» (Behnk Elektronik, Germany) **(Fomina *et al.*, 2017)** using medical kits. To assess the state of plasma coagulation hemostasis, the following parameters were determined: APTT, PT, and TT, using human thrombin (APTT-test, Blood Coagulation Test, Techplastin-test; Technology-Standard LLC, Russia), and quantitative analysis of fibrinogen (Fibrinogen-test; RENAM, NGO of the Russian Society of Hemophilia). Plasma fibrinolytic activity was measured by detecting SFMC in the O-phenanthroline test («SFMC-testplate version»; Technology-Standard LLC, Russia). The analysis was performed according to the manufacturer's instructions.

The values of the outcomes are presented as mean and standard error of the mean (M ± SE). Reliability of differences of blood parameters for multiple independent samples was determined using Kruskal-Wallis one-way analysis of variance.

RESULTS AND DISCUSSION

Secondary hemostasis is mainly performed by plasma clotting factors, and it includes three phases, and the functional state of the studied fish is presented in Table (1). The present results revealed that, APTT was reliably different in sturgeon and carp from the hybrid and the tilapia. APTT of sturgeon was 20.6 times longer than that of the related hybrid and 16.8 times longer than that of the tilapia. APTT of carp was 2.8 and 2.2 times longer, respectively.

Table 1. Parameters of the plasma-coagulation component of hemostasis in commercial fish (the current research)

Parameter	*A. baerii* (n = 12)	*A. ruthenus* × *A. stellatus* (n = 12)	*C. carpio* (n = 30)	*O. niloticus* (n = 14)
TT (sec)	28.02 ± 10.23^{ct}	24.94 ± 5.19^{ct}	500.36 ± 59.42^{sh}	648.38 ± 38.70^{sh}
PT (sec)	241.28 ± 34.99^{c}	176.85 ± 42.67^{c}	457.31 ± 58.32^{sh}	316.96 ± 54.67
APTT (sec)	148.06 ± 54.75^{ht}	7.18 ± 1.40^{sc}	20.12 ± 2.04^{ht}	8.88 ± 1.52^{sc}
Fibrinogen (g/l)	3.02 ± 0.38^{t}	3.79 ± 0.79^{ct}	1.99 ± 0.33^{ht}	0.62 ± 0.01^{shc}
SFMC (mg/100 ml)	17.63 ± 2.51	7.53 ± 1.61^{c}	24.90 ± 1.61^{ht}	14.93 ± 2.17^{c}

Differences are reliable ($p \leq 0.01$): ^{s}– with sturgeon (*A. baerii*);^{h}– with a hybrid (*A. ruthenus* × *A.stellatus*); ^{c}– with carp (*C. carpio*); ^{t} – with tilapia (*O. niloticus*)

When evaluating the extrinsic clotting pathway, which is of predominant importance in fish **(Doolittle & Surgenor, 1962; Sheehan *et al.*, 2001)**, it could be noted that cartilaginous ganoids have 1.9-2.6 times reliably more rapid clot formation (according to PT) when tissue factor is added compared to bony fish.

The fibrinogen quantitative content in blood plasma providing clot formation was the lowest in the tilapia; 4.8-6.11 times lower than in cartilages ganoids, and 3.2 times lower than in carps. Analyzing the obtained characteristics of the plasma-coagulation component of the bony fish clotting system and comparing them with the corresponding ones of cartilaginous ganoids, it can be determined that the rate of fibrin clot formation (TT) in the former is reliably higher by 17.8-26 times than in the latter. Interestingly, they are markers of thrombinemia in human intravascular clotting (disseminated intravascular coagulation (DIC) syndrome) (**Aoki *et al.*, 2018**) and usually do not exceed 3.0 ± 0.1 mg/100 ml **(Momot & Mamaev, 2008)**. The analysis of the obtained data shows that carps have SFMC in 3.3 and 1.7 times reliably more than hybrids and the tilapia, respectively

Based on the revealed species-specificity of hemostasis functioning in fish, it is hardly possible to confirm or deny the theory **(Ivanov, 2021)** related to the cause of such changes in different resistance of fish to stress without the use of handling-stress associated with blood sampling. Our previous studies on the effect of hormonal stress **(Berezina & Fomina, 2020)** confirm the similar effect of short-term handling stress and artificial hormonal stress on the coagulogram.

J. H. Lewis's studies **(Lewis, 1996)** showed some indicators of the functional state of the secondary hemostasis in several species of sharks and rays - typical representatives of cartilaginous fish (*Chondrichthyes*). According to those studies, TT using bovine thrombin was: 57.4 sec (dogfish); 33.8 sec (*Carcharhinus maculipinnis*); 29.8 sec (*Prionace glauca*) and 44.4 sec (*Raja eglanteria*). The extrinsic plasma clotting pathway was studied using different tissue extracts, and it was as follows when using cerebral tissue extract – 8.6 sec (dogfish), 38.4 sec (*Carcharhinus maculipinnis*), 27.8 sec (*Prionace glauca*), 44.4 sec (*Raja eglanteria*); gilled – 10.3 sec (dogfish), 32.4 sec (Carcharhinus maculipinnis), 25.4 sec (*Prionace glauca*), 68.2 sec (*Raja eglanteria*); and skin – 31.4 sec (dogfish), 51.7 sec (*Carcharhinus maculipinnis*), 51.7 sec (*Prionace glauca*), 57.2 sec (*Raja eglanteria*). The activation of the intrinsic clotting pathway (characterized by APTT) was more than 120 sec, which meant that the activation process by the intrinsic pathway in *A. baerii* is closer to cartilaginous fish. Literature data

(Kawatsu, 1986) indicate that the APTT and PT values in carps were much less than the values obtained in the present study.

SFMC are small fragments of blood clots formed during massive thrombosis as a result of the thrombus breaking after the healing of the vascular wall **(Baruzdina & Oshurkova, 2016a, 2016b)**. It is worth mentioning that, SFMC are markers of thrombinemia in human intravascular clotting (DIC syndrome) **(Aoki *et al.*, 2018)**. Nevertheless, they usually do not exceed 3.0 ± 0.1 mg/100ml **(Momot & Mamaev, 2008)**. Meanwhile, SFMC in fishes are 1.3-4.3 times higher than SFMC in dogs **(Baruzdina & Oshurkova, 2016b)**. Furthermore, SFMC is 2.5-8.3 times higher than that recorded in humans **(Momot & Mamaev, 2008)**. However, except for carps whose SFMC is 1.4 times higher, other fish species have 1.0-2.4 times lower SFMC than that observed in cattle **(Fomina, 2009)**.

It should be noted that some signs of a combination of hyper- and hypo-coagulation in carp identified in this study are fundamental for DIC syndrome in humans and mammals **(Aoki *et al.*, 2018; Dubova *et al.*, 2020)**, for which the search for clinical diagnostic tests and therapeutic methods in veterinary ichthyopathology is relevant.

By the basic clinical diagnostic tests, it is possible to conclude that the functioning of the clotting cascade in commercial fish: its activation by the common pathway is several times faster in cartilaginous ganoids than in both species of bony fish. This observation was confirmed with the difference in the level of fibrinogen; its highest amount was detected in cartilaginous ganoids, whereas the lowest was found in the tilapia. Carp also had slower clotting by the extrinsic pathway. Hemostasis, with activation of the intrinsic pathway, was faster in the hybrid and the tilapia, unlike carp and sturgeon. The highest amount of fibrin degradation products was detected in carp, which, along with the slowing of PT and APTT, may indicate the activity of the processes of thrombosis and fibrinolysis with the revealed signs of hypocoagulation due to stress. The lowest SFMC was in the hybrid, which, with the rest of the data, can be explained by the activity of coagulation processes and inactivity of fibrinolysis.

CONCLUSION

Thus, the data obtained confirm the vast variability of coagulation parameters in fish of different classes and fishery importance, for which it is worth considering the identified species specificity when developing and implementing diagnostic and therapeutic methods.

Information on plasma hemostasis in lower vertebrates in general and in commercial fish, in particular, is highly fragmented in the literature. The obtained data revealed some functioning issues of the ancient blood coagulation mechanism in these animals, both in the evolutionary and veterinary aspects. On an applied level, clotting research has the potential to diagnose fish diseases, and further research is necessary to develop practical prophylactic and therapeutic anticoagulation methods for fish farming.

Unfortunately, due to the paucity of data, it is impossible to comprehensively compare the investigated plasma-coagulation hemostasis in bony fish with cartilaginous fish. The authoring team advised to take care when analyzing the conclusions obtained and remember that, at the present moment, neither the developed standardized techniques nor the reagents are available for the study of coagulogram parameters of poikilothermic

animals. It should be remembered that the results also depend on the type of thrombin used, tissue factor and even laboratory dishes. Therefore, further research is recommended to attain more developed results.

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