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REVIEW

A review of the blood coagulation system of fish

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ABSTRACT: (A review of the blood coagulation system of fish). Hemostasis is a function of paramount importance when an animal is responding to an injury or has a blood coagulation disorder. It is important to stop the bleeding after a vascular injury, in order to prevent blood loss, so the body needs an effective coagulation mechanism. In fish, despite the fact that there are many studies about the intrinsic and extrinsic coagulation factors, some significant questions about the regulation of the coagulation system remain unanswered. Therefore, the following review was written to help consolidate the information in the literature so it can be compared and discussed. Several aspects of the coagulation mechanisms in teleost fish, including some of the related substances involved in this process, the factors that are involved in hemostasis, the implications of thrombocytes in the intrinsic coagulation system, and the differences in the methods of measuring blood clotting time are discussed in this review.
Key words: blood, coagulation, fish, hemostasis.

RESUMO: (Uma revisão do sistema de coagulação sanguínea de peixes). Hemostasia é uma função de grande importância para responder a danos e desordens da coagulação sanguínea. Assim, é fundamental para prevenir hemorragia e perda de sangue após um dano vascular, pois o corpo necessita de um mecanismo de coagulação eficiente. Em peixes, apesar de existirem diversos estudos sobre fatores intrínsecos e extrínsecos, algumas questões significantes sobre a regulação do sistema de coagulação permanecem em aberto, uma vez que estes dados estão dispersos na literatura. Esta revisão compara e discute os diversos aspectos dos mecanismos de coagulação em peixes teleósteos, incluindo algumas substâncias relacionadas a este processo, fatores envolvidos na hemostasia, as implicações dos trombócitos no sistema intrínseco da coagulação e diferenças no tempo de coagulação sanguínea.
Palavras-chave: sangue, coagulação, peixe, hemostasia.

INTRODUCTION

Although all multicellular invertebrates have blood cells and a variety of homeostatic mechanisms, the evolution of the vertebrates appears to be an important step in establishing the hematological pattern found in "higher" vertebrates such as mammals. All multicellular animals require efficient hemostatic mechanisms to prevent the loss of fluid (hemolymph or coelomic fluid) and cells following injury, which can occur by three different mechanisms in invertebrates: plasma gelatinization, blood cell aggregation, or a combination of plasma gelatinization and blood cell aggregation (Doolittle 1993, Rowley *et al.* 1997, Aird 2003). In the same way, aquatic vertebrates also need very efficient hemostatic mechanisms to respond to vascular damage and other general injuries. Particularly fish, which have delicate gills where the blood supply is in close proximity to the surrounding environment. These factors render fish exceptionally vulnerable to mechanical gill damage and fish with wounded gills are commonly observed in nature and in aquaculture. Therefore, unless a fish has an effective means to control blood flow to damaged parts of its gills, it will bleed until death when it is injured. Thus, the gills and other areas of the tegument are susceptible to mechanical damage that leads to blood loss (Hill & Rowley 1996, Rowley *et al.* 1997) and also to infection, eliciting the participation of thrombocytes.

Fish thrombocytes are complete cells involved in hemostasis and other activities, unlike the platelets of mammals that are only fragments of cells. Nevertheless, for many years, fish hematologists were not aware that thrombocytes occur in fish. In 1927, Rogers reported that fish blood was deprived of thrombocytes (Srivastava 1969). However, the presence of thrombocytes was confirmed by Jordan & Speidel (1924) and Reznikoff & Reznikoff (1934). Around the same time, Bradley (1937) noted that the blood of some fish contained clotting structures, which he called tigmocytes. Although these structures were called tigmocytes by Bradley, it is possible that they were thrombocytes. Over time, it was demonstrated that the thrombocytes were biological fish cells that were equivalent to the platelets in mammals. In addition, Doolittle & Surgenor (1962) showed that thrombocytes were fragile cells in the teleost *Tautoga onitis* (Linnaeus, 1758), and reasonably stable in the cartilaginous *Mustelus canis* (Mitchill, 1815).

Recent studies have shown that the thrombocytes are involved in hemostasis and organism defense, and are produced mainly by the spleen and kidney in teleost fish. Moreover, studies about thrombocyte ontogeny indicate that they initially appear in the spleen during the first week of postfertilization, while they appear in the blood only after the fourth week of postfertilization (Romano *et al.* 1997). The thrombocytes, when in circulation, possess

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a four-day half-life, but some can survive up to 70 days. This short half-life suggests the need for frequent removal and substitution of these cells to maintain homeostasis and hemostasis (Fischer *et al.* 1998).

Studies of hemostasis in fish began in the last century. According to Doolittle & Surgenor (1962), pioneer studies of Zuns (1933) and Warner (1939) reported differences in clotting among different fish groups. Zuns found that elasmobranchs had frequent hemorrhages due to the presence of little or no blood prothrombin, while Waner reported that blood coagulation in teleosts was fast, and when there was prothrombin conversion, it was not always complete. Wolf (1959) investigated the coagulation time of three trout species, and concluded that the time was much shorter compared to mammals. More complete studies of the fish coagulation system were conducted in 1962 by Russel F. Doolittle from the University of California, San Diego (USA), when he documented that thrombin, from mammals, had efficiently clot fibrinogen in adult lampreys (*Petromyzon marinus* Linnaeus, 1758); however, the inverse was not observed. It was also demonstrated that the blood of the teleost *T. onitis,* when collected directly into glass tubes with no anticoagulant, clots very rapidly and forms firm clots in less than one minute. In contrast, under these same experimental conditions, the blood of the dogfish, *M. canis*, clotted very slowly, and lamprey blood formed a weak, poorly organized clot after 24 hours (Doolittle & Surgenor 1962) at room temperature. During this same year, the evidence for the existence of tissue factor-based coagulation in fish was reported for the first time (Doolittle & Surgenor 1962, Doolittle *et al.* 1962), but it was not confirmed in teleosts until later. In this period, Srivastava (1969) described other important studies about clotting time and the function of thrombocytes for several freshwater teleosts. Other studies were carried out in sharks. These studies demonstrated that, in *M. cannis*, ADP did not lead to the aggregation of thrombocytes (Belamarich *et al.* 1966), unlike adenosine (Kien *et al.* 1971). While in *Heterodontus portusjacksoni* (Meyer 1793), the aggregation and the disaggregation were dependent on the temperature but not thrombin and ADP (Stokes & Firkin 1971).

Surprisingly, little was known about the hemostatic mechanism in teleosts, when only a few elements of the blood coagulation were identified, and fish blood coagulation was fundamentally considered similar to the clotting mechanism in mammals, despite numerous studies (Doolittle *et al.* 1962, Doolittle 1965a, b, Cottrell & Doolittle 1976, Doolittle *et al.* 1976, Doolittle *et al.* 1987, Wang *et al.*, 1989, Doolittle 1993). However, these studies identified prothrombin in the plasma of a species of lamprey (*P. marinus*), smooth dogfish (*M. canis*), and teleost (*T. onitis*), which is an indispensable protein in the coagulation process. It was also demonstrated that calcium, at high concentrations, accelerated the coagulation intrinsic factors in the plasma of smooth dogfish and teleost fish. In the smooth dogfish, the minimum clotting time occurred when there was 50.0–60.0 mmoL/L of calcium, which is about the same as the clotting time for human plasma when it contains its optimum calcium concentration (6 mmoL/L). However, in teleost fish the calcium effect was the greatest when the concentration was 20.0–30.0 mmoL/L. Under these conditions the clotting time was just over one minute, which was the fasted time of the evaluated species (Doolittle & Surgenor 1962).

During this period, studies also documented the existence of thrombin, fibrinogen, tissue factors, and plasminogen in lampreys, in addition to the coagulation factors already described above (Doolittle *et al.* 1962, Doolittle 1965a, b, Cottrell & Doolittle 1976, Doolittle *et al.* 1976, Doolittle & Feng, 1987; Wang *et al.* 1989). However, more complete studies about coagulation factors and mechanisms of fish blood did not take place until later, in spite of the fact that this process is of paramount importance to vertebrates.

BLOOD COAGULATION: INTRINSIC AND EXTRINSIC FACTORS

Fishes are aquatic vertebrates that are members of the largest and most diverse vertebrate taxon, which dates back over 500-million years. They have evolved into three major groups: Agnatha (hagfish and lampreys), Chondrichthyes (sharks, skates, and rays), and Actinopterygii (bony fish). The bony fish diverged from another important group, the Elasmobranchiomorphi, whose recent representatives are the Chondrichthyes (Brum, 1995, Rowley *et al.* 1997, Davdson *et al.* 2003a, b), or cartilaginous fish. A number of important elements in the hematology of vertebrates first appeared in fish, including the evidence of a similar coagulation system. A marked increase in genomic complexity during fish evolution is suggested by the presence of multiple genomes in over 20,000 species. Cartilaginous and bony fish, which probably coevolved from separate ancestors, are different from each other in some aspects, as amphibians are different from reptiles or mammals (Rowley *et al.* 1997). Thus, when comparing teleosts to jawless vertebrates (hagfish and lampreys), jawless vertebrates have a network involving tissue factor, prothrombin, and fibrinogen (Doolittle 1993, Davdson *et al.* 2003a,b), but the tissue-factor pathway involves the VII and X factors, and tissue-factor is the only activating system of the primitive blood coagulation system of cyclostomes (Kimura *et al.* 2009). Hence, they exhibit a clotting time that is slower when compared to sharks and teleosts fish. However, even in cartilaginous or bony fish, it seems that several mechanisms interact to maintain the fluidity of the blood inside blood vessels, without clotting or overflowing, and the combination of these mechanisms creates the equilibrium that corresponds to hemostasis.

Several different kinds of blood coagulation analyses performed on teleosts indicate that the coagulation

process is fundamentally similar to other vertebrates (Woodward *et al.* 1981, Smit & Schoonbee 1988, Lloyd-Evans *et al.* 1994, Pavlidis *et al.* 1999, Jagadeeswaran & Sheehan 1999, Jagadeeswaran *et al.* 2000, Davdson *et al.* 2003a, b, Manseth *et al.* 2004), such as mammals. Moreover, the process found in fish is different from those of amphibians and reptiles, which display a coagulation system similar to the ciclostomes (Davdson *et al.* 2003a, b).

Studies on prostaglandins reported that blood of marine fish coagulates faster than blood of freshwater fish, because the biological activity can vary among species of fish; however, both marine and freshwater fish synthesize prostaglandins in the same way (Matsumoto *et al.* 1989). Nevertheless, studies on the coagulation profile of marine fish suggest that there is no difference between the hemostasis mechanisms of marine and freshwater teleosts (Pavlidis *et al.* 1999).

In the teleosts fish, intrinsic, extrinsic, and common pathway factors of the coagulation system were demonstrated in biochemical studies (Doolittle & Surgenor 1962, Smit & Schoonbee 1988, Jagadeeswaran & Liu 1997, Pavlidis *et al.* 1999, Jagadeeswaran & Sheehan 1999, Jagadeeswaran *et al.* 2000, Davdson *et al.* 2003a,b), in molecular analyses of the blood plasma (Banfield & MacGillivray 1992, Ylönen *et al.*, 1999, Jagadeeswaran & Sheehan 1999, Sheehan *et al.* 2001, Ylönen *et al.* 2002, Davdson *et al.*, 2003a,b, Jiang & Doolittle 2003, Manseth *et al.* 2004), immunologic characterization (Rombout *et al.* 1996, Hill & Rowley 1998, Kfoury Jr. *et al.* 1999, Jagadeeswaran *et al.* 1999), and in studies relating to the morphology and the function of the thrombocytes in different species (Woodward *et al.* 1981, Lloyd-Evans *et al.* 1994, Hill & Rowley 1996, Hill & Rowley 1998, Jagadeeswaran *et al.* 1999, Hill *et al.*, 1999a,b, Grosser *et al.* 2002). Results of these studies suggest that teleosts and mammals are similar, in spite of the significant evolutionary distance between these groups. They also suggest that the main coagulation event in teleosts is the conversion of the catalyzed thrombin, in blood plasma, to fibrinogen, and then the formation of a fibrin clot. Table 1 denotes the name, number, and function of these coagulation system factors known in teleosts, as well as the existence of substances related to this system. However, the names and functions of these factors in teleosts were based on the studies of mammals, which are very well established, because these two groups of vertebrates are relatively similar.

Many of the substances related to blood coagulation in mammals are still unknown in fish, although some have been identified. For example, kininogens are important proteins that participate in blood coagulation intrinsic factors and the substratum for kallikrein was described in the following teleosts: *Salmo salar* Linnaeus, 1758 (Ylönen *et al.* 1999), *Anarhichas minor* Olafsen, 1772, and *Gadus morhua* Linnaeus, 1758 (Ylönen *et al.* 2002). In addition, protein S and C were identified in two species of teleosts, zebrafish (*Danio rerio* Hamilton, 1822) and fugu (*Fugu rubripes* Temminck & Schlegel, 1850) (Jagadeeswaran & Sheehan 1999, Sheehan *et al.* 2001, Jiang & Doolittle 2003, Davidson *et al.* 2003b). Davidson *et al.* (2003a) reported that there was no functional evidence or any cloned sequences in fish with identical sequences of protein S. However, the presence of protein C suggested that protein S was also present. The protein-S gene has been putatively identified in the genome of *F. rubripes*, with a similar intron-exon structure that encodes a protein with 48% sequence identity and 70% sequence similarity with human protein S. Pre-incubation of zebrafish plasma with Protac indicated the presence of a C-like active protein, while unfractionated heparin added to plasma of this fish demonstrated the presence of heparin cofactor activity (Jagadeeswaran & Sheehan 1999).

Antiprothrombin, a member of the serpin superfamily of proteinase inhibitors, is a major inhibitor of the coagulation serine proteinases in mammals and plays a crucial role in the maintenance of normal hemostasis. Antiprothrombin inhibits target proteinases by forming tight equimolar complexes in which the reactive bond of

Table 1. Coagulation factors known in teleost fish, function and possible related substances

Number and/or name	Function
I - Fibrinogen	Gels to form fibrin clot
II - Prothrombin	Active I, V, VII factors and thrombocytes
IV - Calcium (Ca^{++})	Active II factor
V - Proaccelerin	Support to X factor, and active II factor
VII - Stable factor	Active the IX, and X factors
VIII - Antihemophilic factor	Support for IX factor, and active X factor
IX - Christmas factor	Active X factor
X - Stuart-Prower factor	Active II factor
XI - Plasma thromboplastin antecedent	Active XII factor and prekallikrein
XII - Hageman factor	Active XI factor and prekallikrein
High molecular weight kininogen	Supports reciprocal activation of XII factor and prekallikrein
Prothrombinase system, formed by the integration of the factors Xa, Va, Ca^{++} and phopholipids	Active II factor
Antiprothrombin	Inhibit IIa and Xa factors, cofactor for heparin
Protein S	Cofactor for activated protein C
Protein C	Inactive the V and VIII factors

the inhibitor interacts with the active site of the proteinase (Andersen *et al.* 2000). The presence of heparin and dermatan sulfate cofactor activity in zebrafish plasma was confirmed, which would suggest the presence of antiprothrombin inhibitors (Jagadeeswaran & Sheehan 1999). The predicted mature liver antiprothrombin of Atlantic salmon, *S. salar*, consists of 430 amino acids and it has shown approximately 67% sequence identity compared to antiprothrombins of mammals and chickens. Thus despite the long evolutionary distance between fish and humans several features of antiprothrombin were conserved, including the characteristic reactive site of Arg-Ser and six cysteines (Andersen *et al.* 2000). Therefore, antiprothrombin is also present in teleosts, and this has been demonstrated by biochemical and molecular studies.

Coagulation is a complex process in which the blood begins the formation of a solid clot. This is a very important phase of hemostasis, mainly when blood vessels are damaged. Primarily, coagulation begins in a few seconds with the formation of a thrombocyte plug, in the damaged site, when the thrombocytes adheres to the collagen fibers (tissue factor) of the blood vessels using a specific receptor. Soon after, several plasma components designated from coagulation factors respond in a complex cascade to form the fibrin, which reinforces the thrombocyte plug. This is called secondary hemostasis, which can be didactically divided in three compartments: common pathway, intrinsic pathway, and extrinsic pathway (Figure 1). Such pathways are constituted by a series of reactions in which a stable form of a protein is activated and transformed into an enzyme (Tab. 1) that will catalyze the next reaction in the cascade

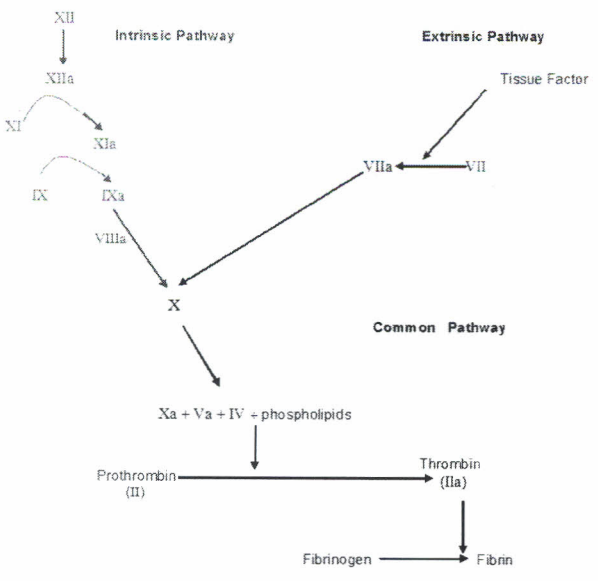

Figure 1. Schematic diagram of the different pathways of blood coagulation in teleosts fish. An international convention recommends the use of roman numeral for the coagulation factors, with fibrinogen as Factor I.

of coagulation. The extrinsic pathway is constituted by the sum of factor VII, which is a unique factor of this pathway. The extrinsic pathway is initiated by exposure of blood to the tissue factor, a specific cellular lipoprotein that was recently confirmed to be present in all vertebrates, including the teleosts (Sangrador-Vegas *et al.* 2002, Davidson *et al.* 2003b). The intrinsic pathway is constituted by the sum of its exclusive plasma factors, which activate those of the final common pathway. The common pathway is reached by completion of either or both of the intrinsic or extrinsic pathways, and results in the elaboration of thrombin (Fig. 1). Therefore, if a coagulation factor that is part of the intrinsic or extrinsic pathway is missing, this deficiency will affect some clinical tests, which will be discussed later on.

Biochemical and molecular studies indicate that blood coagulation involves prothrombin and fibrinogen, besides the tissue factors, in all vertebrates, from humans to primitive lampreys (Doolittle *et al.* 1976, Davidson *et al.* 2003a, b). The blood coagulation network is present in all jawed vertebrates and it evolved before the divergence of tetrapods and teleosts over 430-million years ago (Rowley *et al.* 1997, Davidson *et al.* 2003a,b). From this divergence, blood coagulation in the vertebrates may have benefited from two rounds of gene or whole genome duplication (Davidson *et al.* 2003a,b, Kimura *et al.* 2009). Hence, the fibrinogen molecules are transformed in a clot of soluble fibrin by thrombin (factor Ia) that is derived from a prothrombin molecule (factor II) under mediation of a tissue factor (extrinsic factor) or thrombocyte factor (intrinsic factor) (Doolittle & Surgenor, 1962, Davidson *et al.* 2003a,b, Terra, 2004). In addition, when *in vivo* or *in vitro,* the participation of calcium (factor IV) is essential in this mediation (Doolittle & Surgenor 1962, Terra, 2004). Nevertheless, it is important to point out that *in vivo* factor IX activation is unchained by the complex tissue-factor/factor VIIa, and that the initial activation mechanism of factor IX becomes sustained by factor VIII. Hence, the activation of factor IX seems to be similar to the activation of factor VIII, so that both factors are favored by the thrombin. Therefore, when in the initial phase, when there is no thrombin available, it is possible that the complex tissue-factor/factor VIIa, besides acting directly on factor IX, also acts directly on factor X (Tab. 1 and Fig. 1). Possibly, the small amount of factor Xa obtained contributes to the synthesis of the small amount of thrombin, which is necessary to help in the activation of factor VIII and factor XI, when this phase is elicited in the coagulation process. However, it is also possible that factor XIa is obtained from the activated thrombocytes.

In mammals, including humans, for many years coagulation defects were only analyzed by measuring clotting time and bleeding time. However both methods were abolished for such purpose because current methods, such as the time of fibrin formation in recalcified plasm (Prothrombin Time [PT] and Activated Partial Thromboplastin Time [APTT]), are much more

sensitive and specific. From an execution point of view, these techniques do not differ significantly from the antique methods, except for the fact that they use separate plasma for the analysis. Similarly, all of these methods use the measurement of time as an indicator of the speed of clot formation (Terra, 2004). Such clinical tests exist to evaluate the coagulation factors defects, except for factor VII, which is exclusive from the extrinsic pathway. The clotting time, an in vitro test, is often used for fish because it is quite simple. In this test the fish heart or blood vessel is punctured, the blood is collected in a glass tube without anticoagulants, and then the time of clot formation is recorded. The activation of the clot occurs by simple contact of the blood with the wall of the glass tube, with little participation of the phospholipides on thrombocytes and without the aid of the tissue factor.

For analysis of defects, in the coagulation of fish blood, plasm recalcification time (PRT) can be used, which is an intrinsic system indicator (Fijikata & Ykeda 1985a, c). Since the intrinsic pathway starts by the activation of contact factors in the plasm, the activation can be measured by the APTT; an increase of the APTT reflects the low activity of a factor of this pathway, which could be caused by the absence or or inhibition of the factor. Another test is to measure the time that it takes for prothrombin to form when compared to the concentrations of the factors of coagulation of the extrinsic pathway; hence this test is only useful for inference. However, a simultaneous increase of APTT and PT reflects defects in the common pathway, and they are associated with the low activity of factor VII with any other factor of the intrinsic pathway, such as factors VIII, IX, and XI, or prekallikrein (Terra, 2004). Nevertheless, the methodological conditions (method sensibility) and work environment (temperature, pH, etc.) have an influence on PT, and can interfere in the interpretation of the results. Thus, in *Cyprinus carpio* (Linnaeus, 1758), it was demonstrated that PT is sensitive to temperature variations (Fujikata & Ikeda 1985a, Kawatsu 1986), suggesting that the extrinsic pathway can have thermolabile coagulation factors (Fujikata & Ikeda 1985a, Kawatsu 1986). Therefore, when performing a comparative analysis among species it is necessary that all the conditions previously mentioned are the same.

Hematology is an important aspect of fish physiology, and a relatively large number of reports have described it in the literature. Nevertheless, discussions about thrombocytes and coagulation have been neglected. An analysis of tables 2 and 3 indicates species-specific differences in the coagulation mechanisms in teleost fish. However, for reasons already mentioned, intensive care is necessary when comparing PT data that originated from different studies. Moreover, the differences of each method used for clotting time analysis, among such studies, should also be considered because inconsistent results could be due to these differences. Wolf (1959), when comparing coagulation time using the capillary tube and slide methods, observed that the coagulation time recorded using the slide method was much shorter (Tab. 3). However, these data were compiled from the literature and do not allow for a more precise and reliable analysis, indicating the need for standardized methods for the analysis of blood coagulation in fish. Therefore, as hematological evaluations are gradually becoming important to diagnose the health of fish an appropriated and standardized methodology for analysis of the fish blood coagulation is important and urgently needed.

Blood coagulation time in fish is shorter than in mammals (Wolf, 1959; Doolittle & Surgenor, 1962, Smit & Schoonbee, 1988), due to the high levels of several coagulation factors that contribute to the high activity of the intrinsic and extrinsic pathways (Doolittle & Surgenor, 1962, Smit & Schoonbee, 1988). The extrinsic system in fish seems to be similar to that of mammals; however, the greatest difference between coagulation in fish and mammals is the conversion of prothrombin to thrombin (Doolittle & Surgenor 1962). Nevertheless, the thrombocytes play a central role in the intrinsic coagulation system of fish (Doolittle & Surgenor 1962, Fujikata & Ykeda 1985a,c, Jagadeeswawaran *et al.* 1999) by virtue of their adhesive and aggregative behavior, as seen in the platelets of mammals and other vertebrates. Thus, in fish, there is an important correlation between the number of blood thrombocytes and the clotting time (Tab. 3). This is because fish thrombocytes are sources of indispensable phospholipides that contribute to the activation of the coagulation factors, which activate the conversion of prothrombin to thrombin, which activates

Table 2. Prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen in various healthy marine and freshwater teleosts.

Species	PT (seconds)	APTT (seconds)	Fibrinogen (g/L)	References
Sparus auratus	31.0	47.0	2.8	Pavlidis *et al.*(1999)
Dicentrarchus labrax	12.0	26.0	2.6	Pavlidis *et al.*(1999)
Pagrus pagrus	12.5	25.0	1.8	Pavlidis *et al.*(1999)
Dentex dentex	30.0	58.5	2.5	Pavlidis *et al.*(1999)
Cyprinus carpio	3.8-4.4	6.0-7.4	–	Kawatsu *et al.*(1989)
Cyprinus carpio	31.8	–	–	Fujikata & Ikeda (1985a)
Cyprinus carpio	28.9	12.4	–	Fujikata & Ikeda (1985b)
Cyprinus carpio	23.5	5.1	–	Kawatsu (1986)
Oreochromis mossambicus	21.1	–	1.8	Smit & Schoonbee (1988)
Oreochromis mossambicus	20.9	–	–	Smit & Schoonbee (1988)

Table 3. Mean values of the clotting time (CT) of the blood, and thrombocyte number reported in literature for several healthy freshwater teleosts. Sec. = Seconds, ♀= Female, ♂= Male.

Fish species	CT (seconds)	Thrombocytes (μL)	References
Carassius auratus	10.8	—	Smith *et al.*(1952)
Ictiobus bubalus	41.3	—	Smith *et al.*(1952)
Lepomis macrochirus	29.6	—	Smith *et al.*(1952)
Pomoxis annularis	23.1	—	Smith *et al.*(1952)
Chaenobryttus coronarius	26.8	—	Smith *et al.*(1952)
Ameiurus melas	37.3	—	Smith *et al.*(1952)
Ameiurus natalis	41.5	—	Smith *et al.*(1952)
Onchorhynchus mykiss	42.0	—	Wolf (1959)
Onchorhynchus mykiss	16.5	—	Wolf (1959)
Prosopium williamsoni	72.1	17,600	McKnight (1966)
Colisa fasciatus	37.3	—	Srivastava & Agrawal (1979)
Clarias batrachus	29.6	29,655	Srivastava (1969)
Amphipnous cuchia	19.7	31,600	Srivastava (1969)
Heteropneustes fossilis	27.2	45,773	Srivastava (1969)
Heteropneustes fossilis	17.2	—	Joshi & Sharma (1982)
Channa punctatus	24.4	57,379	Srivastava (1969)
Channa punctatus ♂	40.8	26,200	Yadava *et al.*(1981)
Channa punctatus♀	44.2	25,600	Yadava *et al.*(1981)
Channa striatus ♂	66.0	20,400	Yadava *et al.*(1981)
Channa striatus ♀	67.4	19,600	Yadava *et al.*(1981)
Channa gachua ♂	50.6	22,520	Yadava *et al.*(1981)
Channa gachua ♀	54.8	20,780	Yadava *et al.*(1981)
Oreochromis mossambicus	140.7	—	Smit & Schoonbee (1988)
Oreochromis mossambicus	3.9	—	Nussey *et al.*(1995)
Hybrid tilapia	127.0	—	Ferreira *et al.*(2000)
Salminus maxillosus	56.0-472.0	—	Ranzani-Paiva *et al.*(2000)
Labeo rohita	34.4	—	Siddiqui & Naseem (1979)
Cyprinus carpio	35.0		Smith *et al.*(1952)
Cyprinus carpio	37.6	—	Kawatsu (1986)
Cyprinus carpio	43.0-530.0	—	Ranzani-Paiva *et al.*(2000)
Cyprinus carpio	23.7	—	Sakthivel (1988)

the fibrinogen that stimulates thrombocyte aggregation. Therefore, deviations in the number of thrombocytes and in the coagulation factors influence this process (Ranzani-Paiva *et al.* 2000).

Rapid decline of whole blood clotting time occurs when the number of thrombocytes increases in a fish (Casilas & Smith 1977). Therefore, species with a smaller number of thrombocytes, such as *Prosopium williamsoni* Girard (1856), *Channa striatus* Bloch (1793), and *Channa gachua* Hamilton (1822), have slower clotting times when compared to other species (Tab. 3). Therefore, we can suppose that other species (e.g., *Astronotus ocellatus* Agassiz, 1831; *Rhamdia quelen* Quoy & Gaimard, 1824; armored cathfish [*Loricaria* and *Hypostomus*]; *Prochilodus lineatus* Valenciennes, 1837, and salmonids) would have slow coagulation times, considering they all have a low number of thrombocytes compared to other species (e.g., *Colossoma macropomum* Cuvier, 1818; *Piaractus mesopotamicus* Holmberg, 1887; and *Ictalurus punctatus* Rafinesque, 1818) (Tab. 4). Besides these interspecific variations, Ranzani-Paiva *et al.* (2000) reported that the coagulation time also varies among individuals from the same fish species.

FACTORS INFLUENCING BLOOD COAGULATION MECHANISMS

Clotting time is a method to qualitatively and quantitatively measure several factors interfering with homeostasis mechanisms (Ranzani-Paiva *et al.* 2000). For example, in one species of carp (*C. carpio*), the clotting time (CT) varies from 23.7 to 530.0 seconds (Tab. 3), and a time that exceeds this range indicates a severe deficiency in one or more of the coagulation proteins. When the clot retraction is weak and friable it indicates hypofibrinogenaemia, but if there is early dissolution it can be interpreted as enhanced fibrinolysis. Therefore, changes in clotting time can be an indicator of a haemostatic problem, which could be caused by vitamin deficiency, vascular abnormalities, liver damage (Macnab & Ronald 1965, Holst, 1975, Kawatsu 1986, Van Pittius *et al.* 1992, Ranzani-Paiva *et al.* 2000, Nussey *et al.* 2002, Terra 2004), or other factors. The impact of heavy metals and surface water acidification are serious consequences of environmental pollution. Heavy metals can increase or decrease water pH, interfering with blood coagulation that, together with other physiological disturbances, can cause death (Van Pittus *et al.* 1992). In the case of liver damage this may interfere with the production of fibrinogen, because it is produced in the liver (Terra 2004).

Vitamin K

In 1920, Danish scientist Henrik Dam investigated the role of cholesterol by feeding chickens a chlolesterol-depleted diet, and observed that these animals developed

Table 4. Mean values of total thrombocyte counts (μL) for several healthy freshwater teleosts.

Species	Mean values	References
Rutilus rutilus	2.000	Catton (1951)
Salmo trutta	2.000	Catton (1951)
Salmo trutta	14,000	Pickering (1986)
Salmo trutta	20,000	Pickering (1986)
Ictalurus punctatus	68,400	Grizzle & Rogers (1985)
Ictalurus punctatus	77,870	Tavares-Dias *et al.*(2007)
Pimelodus maculatus	32,600	Ribeiro (1978)
Rhamdia quelen	11,300	Forresti *et al.*(1977a)
Hoplias malabaricus	2.900	Forresti *et al.*(1977b)
Hoplias malabaricus	50,600	Camargo *et al.*(1986/87)
Carassius auratus	46,100	Murray & Burton (1979)
Cyprinus carpio	47,900	Kozinska *et al.* (1999)
Cyprinus carpio	12,600	Imagawa *et al.*(1989)
Cyprinus carpio	47,075	Tavares-Dias (2003)
Cyprinus carpio	25,490	Tavares-Dias *et al.*(2004)
Cyprinus carpio	30,940	Tavares-Dias *et al.*(2004)
Prosopium williamsoni	17,600	McKnigth (1966)
Chirostoma sp.	27,630	Alaye-Rahy (1993)
Astronotus ocellatus	9,070	Pitombeira (1972)
Oreochromis niloticus	61,690	Ueda *et al.*(1997)
Oreochromis niloticus	48,900	Tavares-Dias (2003)
Hybrid Oreochromis	63,110	Tavares-Dias (2003)
Hybrid Oreochromis	52,760	Hrubec *et al.*(2000)
Hypostomus paulinus	16,570	Satake *et al.*(1986a)
Loricaria macrodon	14,290	Satake *et al.*(1986b)
Hypostomus regani	17,310	Satake *et al.*(1991)
Hypostomus regani	22,710	Satake *et al.*(1991)
Onchorhynchus mykiss	2.800	Lamas *et al.*(1994)
Liposarcus anisitsi	22,100	Cavalcante *et al.*(1995)
Colossoma macropomum	74,750	Tavares-Dias (2003)
Piaractus mesopotamicus	70,400	Tavares-Dias (2003)
Piaractus mesopotamicus	56,580	Tavares-Dias & Mataqueiro (2004)
Hybrid tambacu	49,240	Martins *et al.*(2001)
Hybrid tambacu	45,800	Tavares-Dias (2003)
Brycon amazonicus	66,320	Tavares-Dias (2003)
Brycon orbignyanus	25,740	Tavares-Dias (2003)
Brycon amazonicus	30,692	Tavares-Dias *et al.* (2008)
Leporinus macrocephalus	47,870	Tavares-Dias (2003)
Prochilodus lineatus	21,410	Tavares-Dias (2003)
Ictalurus punctatus	78,950	Tavares-Dias (2003)

hemorrhages and started bleeding under these conditions. Adding purified cholesterol to the diet did not stop the hemorrhages and, thus, it appeared that, together with the cholesterol, a second compound was missing from the food. This second compound was called the coagulation vitamin. This new vitamin received the letter K because the initial findings were reported in a German journal, in which it was designated as "Koagulations Vitamin." However, the precise function of vitamin K was unknown until 1974, when the vitamin K-dependent coagulation factor prothrombin was isolated from a bovine, which had received a high dose of the vitamin K antagonist, warfarin.

Vitamin K is considered an essential nutrient for all animals. However, its essential role as a vitamin in fish has been little studied. Recent studies with the teleosts *D. rerio* (Jagadeeswaran *et al.* 2000, Sheehan *et al.* 2001) and *F. rubripes* (Jiang & Doolittle 2003) reported the presence of the genes for vitamin K dependent factors, such as factors VII, IX, X, and prothrombin. In *C. carpio*, it was also reported that warfarin prolonged the PT and APTT but the supplementation of vitamin K prevented the increase of PT until the doses of warfarin reached 1.0 mg (Kawatsu *et al.* 1991). However, in *D. rerio* warfarin inhibited the gamma-carboxylation of the glutamic acids to prothrombin (Jagadeeswawaran & Sheehan 1999, Jagadeeswawaran *et al.* 2000, Hanumanthaiah *et al.* 2001). Whereas vitamin K-dependent gamma-carboxylation is required for the functional activity of coagulation proteins such as factors VII, IX, X, and prothrombin (Hanumanthaiah *et al.* 2001). Hence vitamin K is essential for the coagulation cascade of the intrinsic and extrinsic pathways. It was also found that vitamin K deficiency causes a reduction of the blood coagulation time in salmonids (Poston 1964, Lovell 1989), and continuous exposure to warfarin for up to 10 to 14 days causes spontaneous bleeding, which was visible in the posterior portion of the body (the tail fin) in a study that used *D. rerio* (Jagadeeswawaran & Sheehan 1999). In a study by Kawatsu *et al.* (1989), carps (*C. carpio*) displayed an increase in the PT and APTT after exposure to molinate, an herbicide. Thereafter, the prolongation of PT became more pronounced than APTT. The prolongation of PT was corrected by the addition of

stored serum, suggesting that low amounts of coagulation factors existed in the serum; hence, these facts suggested that the hemostatic disorders resulted from a significant depression of vitamin K-dependent factors (Kawatsu *et al.* 1989). On the other hand, no change was observed in the coagulation time and prothrombin time of *I. punctatus* when fed a diet without the vitamin K-supplement. This suggests that the catfish, *I. punctatus*, has an extremely low requirement for vitamin K (Murai & Andrews 1977). Nevertheless, the vitamin K requirement seems to be different among species. Although this requirement is not known for fish, it is thought that vitamin K is necessary for fish during hemostasis. For example, Lovell (1989) observed hemorrhages in the skin of *I. punctatus* when its diet was deprived of this vitamin.

Stress

When environmental changes that create stress are maintained, fishmanifest a group of signs known as adaptation general syndrome (Selye 1950), which is divided into stages of alarm, resistance, and exhaustion. Such alterations can be either of biochemical or physiological origin, and they depend on the fish species, besides the stress agent. Activation of hemostatic mechanisms in fish has also been noted following a period of stress and these include a rapid decline of the whole blood clotting time with a concordant increase in the number of thrombocytes present (Wedemeyer *et al.* 1976, Casilas & Smith 1977). In *Oncorhynchus aguabonita* Jordan, 1892, thrombocytes counts were significantly higher in fish anesthetized with tricaine than in unanesthezed fish that were immobilized (Hunn *et al.* 1992). Thus, because alterations in the clotting time are considered a secondary stress effect (Barton & Iwama 1991), the clotting time for whole blood may be a sensitive indicator of environmental stress (Casilas & Smith 1977) in fish.

The stress produced by capturing and handling *Onchorhynchus mykiss* Walbaum, 1792, appeared to have reduced the fibrinogen levels in the blood of the fish (Bouck & Ball 1966), and caused a decline in the fibrinogen levels and an increase in the number of circulating thrombocytes in *Labeo umbratus* Smith, 1841 (Hatting & Van Pletzen 1974). The handling of stress elicited a reduction of the clotting time and an increase of the number of blood thrombocytes in *O. kisutch* and *O. gorbuscha* (Cassilas & Smith 1977), and it also caused a rapid decrease of the plasma recalcification time (PRT), PT, and APTT in *C. carpio* (Fukikata & Ikeda 1985b). Such results corroborate that clotting time is very useful as a stress indicator. In addition it indicates that a decrease in the clotting time is possibly due to an increase in the number of blood thrombocytes, induced by an increase of catecholamines and cortisol, which are produced during stress.

Evaluation of blood coagulation in *Oreochromis mossambicus* (Steindachner, 1864), by determining the whole blood clotting time for traumatic and atraumatic blood samples, showed that the clotting time was 22.7 seconds, while in glass tubes it was 140.7 seconds and in polycarbonate tubes it was 433.5 seconds (Smit & Schoonbee 1988). In *O. mykiss,* after a short period of stress, the number of blood thrombocytes tripled and blood coagulation was always observed (Cassilas & Smith 1977). On the other hand, although clotting time of this same salmonid was reduced as the levels of stress increased, coagulation frequently failed to occur when the fish were not stressed (Ruis & Bayne 1997). Such unlikely results for the clotting time from the same species could be due to the different methods used during the analysis, considering that in this method the activation was induced by the contact of the blood with the glass tubes, with a little aid from the phospholipids of the thrombocytes. Moreover, Cassilas & Smith (1977) used glass tubes to collect fish blood, after cannulation, to analyze the clotting, while Ruis & Bayne (1997) used polypropylene tubes and collected blood directly from blood vessels. Recent studies have shown that coagulation occurs more quickly in glass tubes than in polypropylene tubes (Smit & Schoonbee 1988, Ruis & Bayne 1997). In addition, cannulation probably induced an acute-phase response, altering plasma levels of several factors, which could be involved in the coagulation cascade (Ruis & Bayne 1997); therefore, clotting time for whole blood is a sensitive indicator of the physiological state of fish. However, the standardization of the methods used to analyze clotting time is of utmost importance because it would allow all new studies to be accurately compared to data previously reported in the literature.

Studies on the effects of stressin *C. carpio* demonstrate a decrease in PRT and an increase of the number of thrombocytes in the blood, but prothrombin levels were not affected. These changes were caused by cathecholamines by stress (Fujikata & Ikeda 1985b,c). As PRT is an indicator of the intrinsic system, these results suggest that thrombocytes participate in intrinsic blood coagulation when the level of blood cathecholamines increases, particularly adrenaline in stressed fish that have blood reacting to the adrenergic α-receptor (Fujikata & Ikeda 1985b). Moreover, it also shows that the PRT is useful as a stress indicator, and therefore it can be used to investigate the clotting time mechanisms in stressed fish.

Blood thrombocytes

The number of thrombocytes (2.000-78,900μL) varies among healthy fish species. Intra-specific variations (Tab. 4) are also observed, and are attributed to many factors, both biotic (such as age, season, maturity) and abiotic (including water temperature, pH, dissolved oxygen content, sex, and maturity stage) and in particular to stress (Tavares-Dias & Moraes 2004, Tavares-Dias & Moraes, 2007a, Pavlidis *et al.* 2007, Tavares-Dias *et al.* 2008).

Evaluations of the coagulation system that study the changes in the number of thrombocytes are an important

contribution to understanding haemostasis of fish, considering that such changes may be caused by several factors. For example, when there is thrombocytopenia in fish exposed to water with different chemical products (Grizzle 1977, Chakrabarty & Banerjee 1988a, Van Pittius *et al.* 1992, Nussey *et al.* 1995, Tavares-Dias *et al.* 2002, Nussey *et al.* 2002), or anesthetics (Hunn *et al.* 1992), an increase in clotting time was reported (Kawatsu *et al.* 1989, Pittius *et al.* 1992, Nussey *et al.* 1995). However, in cases when thrombocytosis occurred (Agrawal *et al.* 1978, Srivastava & Agrawal 1979, Dick & Dixon 1985, Khangarot *et al.* 1999) a decrease in clotting time was reported (Srivastava & Agrawal 1979, Nussey *et al.* 2002). When exposed to organophosphorate pesticides, the clotting time and number of blood thrombocytes in *Channa punctatus* fluctuated significantly, and normal values only recovered after three days of exposure (Chakrabarty & Banerjee 1988b) to the chemical product. Carps (*C. carpio*), when exposed to the herbicide molinate, showed an increase in the PT and APTT (Kawatsu *et al.* 1989). Therefore, it is possible that the clotting time and number of thrombocytes also increased.

In *Tor putitora* Hamilton, 1822 (Sharma & Joshi 1985) and *Fundulus heteroclitus* Linnaeus, 1766 (Gardner & Yevich 1969), which are temperate species, the number of thrombocytes increased during the winter, while in *Cirrhinus mrigala* Bloch, 1795, a tropical species, the number of these cells increased during the summer (Raizada & Singh 1981). Therefore, temperature has a different effect on clotting time between temperate and tropical species, because the blood clots more quickly when the temperature is lower (Kawatsu 1986). Elevation of the temperature of the environment increased the number of blood thrombocytes in *C. punctatus* (Dheer 1988) while it produced the contrary effect in *Salmo trutta lacustris* Linnaeus, 1758 (Rahkonem & Pastemack 1998) and *Hippoglossus hippoglossus* Linnaeus, 1758 (Langston *et al.* 2002). Therefore, the number of circulating thrombocytes in fish varies depending on water temperature (McLeay 1975), seasonality (Yadava *et al.* 1981, Alcorn *et al.*, 2002), sex (Yadava *et al.* 1981), and other factors.

In *O. aguabonita* the thrombocyte counts were not different between sexes, but they were significantly higher in fish anesthetized with tricaine than in unanesthezed fish that were immobilized (Hunn *et al.* 1992). In fish, the hypophysis is a gland that is associated with variety of physiological and biochemical processes (Hoar & Randal 1969), and a hypophysectomy in *Heteropneustes fossilis* Bloch, 1794, reduced erythrocytes and leukocytes numbers but prolonged the clotting time of the blood (Joshi & Sharma 1982). The hypophysis influences hematopoiesis (Rasquim 1951) and the erythrocytes and leukocytes values in *F. heteroclitus* (Pickford *et al.* 1971). Therefore it probably influences blood thrombocyte values in fish.

Most species of cold water fish incorporate significant amounts of eicosapentaenoic acid (EPA, 20:5(n-3) in their cell membranes in addition to arachidonic acid (AA), and nutrition received from artificial sources is very important in the maintenance of the health of fish when they are in culture. The production of both AA and EPA has been investigated in fish (Llooyd-Evans *et al.* 1994). Lipids are an important diet component acting as a source of energy and as a source of essential fatty acids that fish cannot synthesize but are needed for basic functions (Klinger *et al.* 1996). Among such functions, are the maintenance of healthy tissues and functional haemostasis, whereas lipids are needed to maintain the structure and function of the cellular membrane (Figs. 2 and 3).

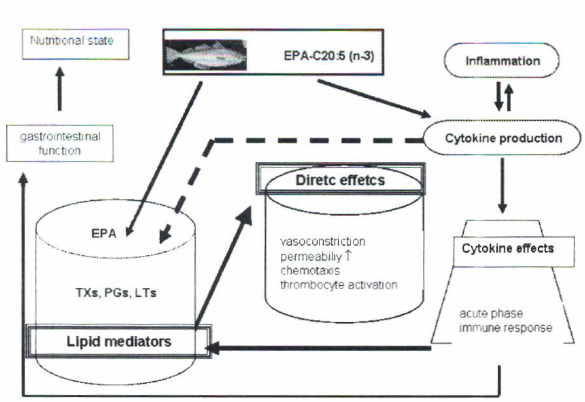

Figure 2. Schematic diagram of effects of omega-3 fatty acid ratio on the eicosanoids regulation (adapted from Heller *et al.*, 2003). EPA=eicosapentaenoic acid, TXs = Tromboxanes, PGs = Protaglandins, LTs = Leukotrienes

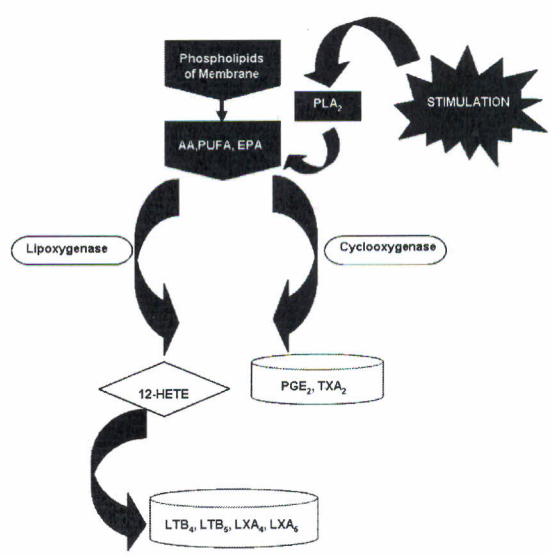

Figure 3. Schematic diagram of products of the metabolism of arachidonic acid (AA), polyunsaturated fatty acid (PUFA), and eicosapentaenoic acid (EPA) via cyclooxygenase and 12-lipoxygenase after activaction of phopholipase A2 (PLA2) in fish thrombocytes (Based on: Lloyd-Evans *et al.*, 1994, Hill *et al.*, 1999a, b, Grosser *et al.*, 2002). 12-HETE= 12-hydroxyeicosatetraenoic, PGE= Protaglandin E2, TXA= Tromboxane A2, LTB4 and LTB5= Leukotriene B4 and B5, LXA4 and LXA5= Lipoxin A4 and A5.

In *Salmo salar*, the time of blood clotting was reduced when the fish received a diet with high sources of n-3 fatty acids and vitamin E (Waagbo *et al.* 1993). When individuals of *C. carpio* were fed diets containing low (14%) and high (58%) protein levels a reduction in clotting time and percentage of blood thrombocytes were reported (Sakthivel 1988). On the other hand, juvenile channel catfish that were fed with diets containing the highest sources of n-3 fatty acids (menhaden oil) exhibited a higher number of thrombocytes and a shorter PRT compared to fish that were fed diets containing lower sources of n-3 fatty acids (beef tallow), which exhibited a longer PRT and a lower number of thrombocytes. Conversely, a diet of soybean oil produced a low count of thrombocytes and low PRT. It has also been found that the level of n-3 in a diet influences the clotting factors and the osmotic fragility of thrombocytes (Klinger *et al.* 1996). Diets containing high levels of certain n-3 fatty acids interfere with the production of thromboxanes, leukotrines, and prostaglandins (Figs. 2 and 3), which are the eicosanoids responsible for the aggregation mechanisms of thrombocytes (Kayama *et al.* 1985, 1986, 1987, Matsumoto *et al.* 1989, Lloyd-Evans *et al.* 1994, Hill *et al.* 1999b, Heller *et al.* 2003) and for the mediation of inflammation (Heller *et al.* 2003). Hence, in recent years interest has been centered on the possible therapeutic use of fish oils rich in polyunsaturated fatty acids (PUFAs).

In mammals, the origin of physiological investigations of n-3 fatty acids, conducted in the early 1970s, and the effects described in these studies were mainly discovered in relation to hemostasis and the function of platelets. Omega-3 PUFA affects the biophysical characteristics of cellular membranes by altering the phospholipid membrane composition and the cholesterol content, which improves membrane fluidity (Heller *et al.* 2003). Moreover, there is evidence that dietary enrichment in long-chain n-3 fatty acids results in strong hypocoagulation. This effect of n-3 fatty acids on anticoagulant activity is mediated by the inhibition of vitamin K-dependent coagulation factors and is specific to the long-chain components. This did not occurr with the linolenic acid, which is present in some vegetable oils (Leray *et al.* 2001). However, the impact of fatty acids on lipid mediator generation is still not understood in fish, hence a complete understanding of the subcellular effects is still unknown. Even so, it seems that a lipid-rich diet affects not only the number but perhaps also the function of thrombocytes. The blood clotting time is influenced by the production of eicosanoids, compounds that are formed from the phospholipids of cell membranes, including the thrombocytes, by the metabolism of arachidonic acid (see Fig. 3). Previous studies suggest that thrombocytes are important to the conversion of prothrombin to thrombin and to clot retraction (Doolittle & Surger 1962, Fujikata & Ikeda 1985a-c). For example, Srivastava (1969) and Yadava *et al.* (1981) found that in several freshwater fish blood coagulated in a shorter time when the number of thrombocytes increased.

In addition to the factors previously mentioned, blood coagulation can be influenced by other factors, such as parasitic infections. For example, when *P. mesopotamicus* was infected with *Argulus* sp. thrombocytopenia was reported (Tavares-Dias *et al.* 1999), and similar results were also found in *O. kisutch* (Lester & Budd 1979), *Salmo trutta* (Rahkonem & Pastemack 1998), *F. rubripes* (Guitang 1998), and *P. mesopotamicus* (Tavares-Dias *et al.* 1999) that were infected with different parasites. However, in *Oreochromis niloticus* Linnaeus, 1758, with ichthyophthiriasis and saprolegniosis (Tavares-Dias *et al.* 2002), and *Leporinus macrocephalus* Garavello & Britski, 1988, infected with the nematod *Goezia leporinus* Martins & Yoshitoshi, 2003 (Martins *et al.* 2004), only a tendency of thrombocytopenia was found. Increase in the clotting time was reported in *Onchorhyncus nerka* Walbaum, 1792, infected with a virus (Watson *et al.* 1956), and hybrid tilapia with saprolegniosis (Ferreira *et al.* 2000), which was probably due to a reduction of the number of circulating thrombocytes. Schuwerack *et al.* (2001) reported that the percentage of thrombocytes decreased in haemopoietic tissues of carps when the fish were infected with *Sanguinicola inermis* Plehn, 1905. In *S. salar*, the Hitra-disease caused a partial increase in time values of prothrombin and thromboplastin, and a reduction of the plasma fibrinogen levels and number of thrombocytes, indicating a coagulopathy with a dissemination of intravascular coagulation (Salte & Norberg 1991). On the other hand, in rainbow trout (*O. mykiss*) an experimental infection with *Renibacterium salmoninarum* provoked an increase in the number of thrombocytes (Bruno & Munro 1986). In *O. keta* that were infected with viral erythrocytic necrosis (ENV), efficiency of clotting decreased progressively over time and no clotting occurred after four months. Even when considering the reduction of thrombocytes in haemopoietic tissue the coagulopathy could be mediated independently of thrombocytes, as a result of deficiencies in serum components of the clotting system (MacMillan *et al.* 1989). However, the possibility that cell destruction was immunologically mediated was not examined. Moreover, the greater tendency of infected fish to die from pathogens suggests that the number of functional thrombocytes decreased.

THROMBOCYTES AGGREGATION

Arachidonic acid (AA) is an important derivative of unsaturated fatty acid, which is part of the phospholipid membrane of cells, including the thrombocytes. A series of important substances with biological properties that are denominated eicosanoids are metabolized from polyunsaturated fatty acid (PUFA), AA, and eicosapentaenoic acid (EPA). Therefore, precursors of the eicosanoids are the lipid compounds of cellular membranes. The type of eicosanoid formed depends on the enzyme pattern of the respective cells. Two principal

pathways are involved in the production of eicosanoids and catalyzed products derived from lipid compounds in thrombocytes (see Fig. 3) and other cells. Such eicosanoids are found in the "higher" invertebrates to mammals (Rowley *et al.* 1997, Pope & Rowley 2002) and play an important role in fish hemostasis. The eicosanoids are "local mediators" and may be generated in most tissues (endothelial cells, keratinocytes) and leukocytes (mononuclear phagocytess, mast cells, neutrophils, eosinophils, and thrombocytes). Production of both AA and EPA derived eicosanoids by thrombocytes has been investigated. Fish thrombocytes can produce a variety of prostaglandins (PGs) and tromboxane B_2 (Fig. 3) that are synthesized by thrombocyte AA (Kayama *et al.* 1985, 1986, 1987, Matsumoto *et al.* 1989, Grosser *et al.* 2002), and tromboxane B_3 from EPA (Kayama *et al.* 1986, 1987). In addition, the formation of lipoxins by 5 and 12-lipoxigenase in thrombocytes of fish has also been reported (Lloyd-Evans *et al.* 1994, Rowley *et al.* 1997).

Cyclooxygenase is continually expressed and its pathway is primarily responsible for the prostaglandins (see Fig. 3), which maintain the homeostatic function (Grosser *et al.* 2002). Hence, the metabolism of lipid compounds by the cyclooxygenase generates the thromboxane (TX) A_2 (see Figs. 2 and 3) and it has an effect on vasodilation, in addition to thrombocyte aggregation (Hill *et al.* 1999b, Pope & Rowlew 2002, Heller *et al.* 2003). The prostaglandin (PG) E_2 has a vasodilatory effect, that modulates the effects of agents that increase vascular permeability in both immune reactivity and inflammatory responses (Knight *et al.*, 1993, Rowley *et al.*, 1997, Zou *et al.*, 1999, Heller *et al.*, 2003). Leukotrienes (LT) B_4 and B_5 increase vascular permeability, and attract neutrophils and other cells via chemotactic properties (Knight *et al.* 1993, Heller *et al.* 2003). Lipoxins (LX) A_4 and A_5 are chemotactic (Knight *et al.* 1993) and chemokinetic for leukocytes (Sharp *et al.*, 1992) and have aggregatory potential (Lloyd-Evans *et al.* 1994). The major product of the lipoxigenase that is derived from AA is 12-hydroxyeicosatetraenoic/12-HETE (see Fig. 3) (Lloyd-Evans *et al.* 1994), which has an effect on thrombocyte aggregation (Rowley *et al.* 1997), and enhances phagocytosis (Knight *et al.* 1993). Cyclooxygenase or lipoxygenase in the cells of many species, including the fish, may generate all of these eicosanoids (see Fig. 3), which are very important for the hemostatic potential and homeostasis. According to recent findings the action of the eicosanoids occurs via receptors on the cells (Hill & Rowley 1996, Bowden *et al.* 1997).

In mammals, diets rich in long-chain n-3 fatty acids influence the physiology, including the improvement of membrane fluidity (Heller *et al.* 2003). In fish, however, this has not been studied until recently, and these studies concentrate mainly on the effects of this type of diet on hemostasis. In *I. punctatus*, a diet with a high amount of fatty acid appears to inhibit or reduce the production of prostaglandins, which are responsible for the aggregation of thrombocytes, and then as a form of compensating for the decrease of prostaglandins there is an increase in thrombocytes (Klinger *et al.* 1996). In the gill filaments of *O. mykiss,* a great variety of ecosanoids are produced (Knight *et al.* 1995, Holland *et al.*, 1999). It was found in another study that after the injury of tissue or a blood vessel the ecosanoids play an important role in the hemostasis (Sundin & Nilsson (1998), stimulating vasoconstriction and thrombocyte aggregation (see Fig. 4), when the thrombocytes display a great diversity in cell shape, from rounded to splindle-shaped forms (Woodward *et al.* 1981, Lloyd-Evans *et al.* 1994, Hill & Rowley 1996, Jagadeeswaran *et al.* 1999).

The blood coagulation system of fish is very complex and involves thrombocytes and additional compounds. In rainbow trout, a strong aggregation of thrombocytes was demonstrated in the presence of collagen (Rombout *et al.*, 1996, Köllner *et al.* 2004), trout fibrinogen (Woodwards *et al.* 1981, Lloyd-Evans *et al.* 1994, Hill & Rowley 1996, Rowley *et al.* 1997, Jagadeeswaran *et al.* 1999), and U-46619, a TX mimetic (Kfoury Jr. *et al.* 1999). Similar to the platelets of mammals, fish thrombocytes need fibrinogen to aggregate. A greater amount of aggregation occurred in the presence of fish (55%) fibrinogen rather than human (15%) fibrinogen, however this aggregation response was inhibited by pre-incubation of thrombocytes, specifically the tromboxane A_2 receptor antagonist. Moreover ultrastructure and aggregate studies of thrombocyte aggregation after incubation with U-46619 revealed clear differences between the amount and dynamics of thrombocytes clumping in the presence of human fibrinogen, compared with trout fibrinogen. Thrombocytes rapidly underwent shape change and aggregation after only one minute without the initial involvement of any other cell types. The maximum

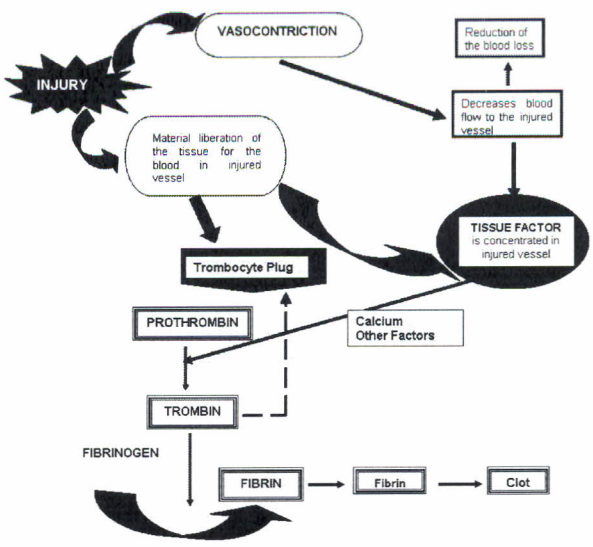

Figure 4. Schematic diagram of homeostasis for teleost fish after tissue injury.

degree of aggregation was achieved after four to eight minutes and a larger aggregation formed in the presence of *O. mykiss* fibrinogen compared to human fibrinogen (Lloyd-Evans *et al.* 1994, Hill & Rowley 1996).

Fibrinogen, as an inactive precursor of the fibrin (see Tab. 1 and Fig. 1), is present in sufficient concentrations in the blood, and can be supplied in great amounts when needed (for example, when a vessel is injured). Moreover, in an injured vessel there are elevated levels of the fibrinogen activator, thrombin (Terra 2004, Miller 2005). Therefore, after a vascular injury, the thrombocytes in fish participate in the formation of thrombin along with the thrombin (see Fig. 4). Many studies have reported (Woodward *et al.* 1981, Lloyd-Evans *et al.* 1994, Hill & Rowley 1996, 1998, Rowley *et al.* 1997, Jagadeeswaran & Liu 1997, Jagadeeswaran & Sheehan 1999, Kfoury *et al.* 1999, Gregory *et al.* 2002) the ability of thrombin to aggregate the thrombocytes on which the fibrin can be deposited, reflecting the interaction among the cellular and enzymatic components of the blood coagulation system (as found in the platelets of mammals). However, because thrombin does not have vasoactive ability, differences exist between fish and mammals regarding the control of vascular homeostais (Rowley *et al.* 1997), but thrombin plays a central role in the coagulation cascade. Thrombin is the pivotal serine protease that converts fibrinogen to fibrin in the coagulation cascade. Thrombin is generated from prothrombin following activation by factor Xa in the presence of cofactor Va during vascular injury. In addition, when converting fibrinogen to fibrin (see Figs. 1 and 4), thrombin is involved in multiple activities. Thrombin alternatively regulates fibrin accretion as an anticoagulant by binding to the endothelial receptor thrombomodulin to convert protein C to activated protein C that inactivates cofactors Va and VIIIa required for fibrin generation (Day *et al.* 2004). The mechanisms that involve hemostasis in fish are relatively similar to the mechanisms in mammals, however they seem simpler. In mammals, when a vascular injury exists, three direct effects occur (Terra 2004). However, in fish, these effects appear to be duplicated (Fig. 4). The first effect is vasoconstriction, which is local. The second effect is blood exposure to the connective tissue and to collagen fibers in the blood vessel walls, increase of vascular permeability with inflammatory cell migration, and the formation of a fibrin clot. In this event thrombin is the compound directly involved in the enzymatic formation of the fibrin clot; it also plays an important role in tissue repairing mechanisms. This causes aggregation of fish thrombocytes (Dolittle *et al.* 1962, Woodward *et al.* 1981, Loyd-Evans *et al.* 1994), as well as in platelets of amphibians, reptiles, birds, and mammals (Belamarch *et al.* 1966, Banfield & MacGillivray 1992). Studies to provide evidence of fibrin formation at the site of vascular injury, in zebrafish, localized fibrin deposits at the site of the thrombus formation and also found thrombocytes adhering to and aggregating on the vessel wall at the site of the injury (Gregory *et al.* 2002). After a vascular injury, during the formation of the thrombus (Fig. 4), both mature and young thrombocytes are present. However, young thrombocytes initiate arterial thrombus formation, when they first adhere to the subendothelial matrix and are rapidly activated, releasing agonists, and also recruiting additional young thrombocytes, which further release more agonists (Thattaliyath *et al.* 2005). Therefore, fish hemostasis is an important physiological defense mechanism that occurs to prevent blood loss (following vascular injury), like hemostasis in mammals, and fish thrombocytes are considered a phylogenetic precursor of platelets (Rowley *et al.* 1997).

Interestingly, in rainbow trout, the immediate hemostatic response to a cut in a gill filament artery is local vasoconstriction, which ceases the hemorrhage within about 20 seconds. After 8 minutes blood coagulation takes over the responsibility for hemostasis and then the vasoconstriction subsides. The antagonists of acetylcholine, adenosine, and serotonin receptors are unable to block the hemostatic vasoconstriction. Moreover, tetrodotoxin presents no effect, indicating a non-nervous origin (Sundin & Nilsson 1998). Therefore, in teleosts, a possible mechanism involves the release of endothelium-derived factors for injury repair, such as the cyclooxigenase products of the achidonic acid metabolism (eicosanoids) and the endothelium.

In fish, collagen is one of the most effective aggregating agents (Woodward *et al.* 1981, Jagadeeswaran *et al.*, 1999) and it resists to inhibition by the adenosine, acetylsalicylic acid (ASA), and prostaglandin (PG) E_1, and to ASA inhibition of arachidonic acid (Jagadeeswaran *et al.* 1999). Moreover, ASA, as with others drugs (dexamethasone and penilicin), affects the collagen biosynthesis in teleosts, interfering with the transcription of the fibroblast genes necessary for the synthesis of elastoidin, or it alters the typical rapid turnover of this protein, thereby affecting regeneration, and this results in disorganization of the collagen (Bechara *et al.* 2002). Drugs that disrupt the collagen metabolism are responsible for the appearance, progression, and persistence of many alterations caused by disordered collagen synthesis, maturation, and spatial organization in the regeneration (Bechara *et al.* 2000) of the tissue of teleosts. This strong response of the collagen to AS, PGE_1, and adenosine may reflect an essential adaptation in fish coagulation because blood cells and clotting factors flowing from an external wound are quickly carried away from the injury by water flowing past the fish (Woodward *et al.* 1981). Furthermore, as a conservation of major hemostatic pathways, thrombocytes and other factors also participate (Jagadeeswaran *et al.* 2002). The use of anti-aggregatings influences fish hemostasis, probably influencing the bleeding time but not the clotting time. This demonstrates that the thrombocytes have a different influence on bleeding time compared to clotting time. When measuring the bleeding time the thrombocytes are activated. When measuring the clotting time, when there is a lack of collagen in the thrombocyte activation,

thrombocytes have a reduced role, and their influence would be almost worthless during clot formation. These results also suggest a reduced initial participation of thrombocytes on the integration effect of the fibrin and other coagulation factors.

Collagen is ubiquitously distributed among the vertebrates, is an important component in a variety of tissues, and provides the structural integrity and the support in blood vessels and tissues. Tissue or vessel injury typically exposes free collagen fibers, which provides a strong stimulus for thrombocyte aggregation, the first step in clot formation and subsequent tissue repair. The repair is preceded by a series of events. At first, in the injured vessels or tissues, vasoconstriction occurs quickly in an attempt to reduce blood flow and to minimize blood loss until the thrombocytes and several factors of the coagulation system buffer the situation. Such an event continues until the fibroblasts appear (fibroblasts can synthesize collagen) and there is dissolution of the hemostatic buffering. For efficient hemostasis to take place the integration of factors and the chronological sequence of several hemostatic mechanisms are necessary. Thus, besides what is described above, other compounds also participate in hemostasis. Aggregation of piscine thrombocytes has been successfully induced by several substances including U-46619 tromboxane mimetic, ADP, ATP, collagen, adrenaline, and thrombin (Woodward *et al.*, 1981, Lloyd-Evans *et al.*, 1994, Hill & Rowley, 1996, Kfoury Jr *et al.* 1999, Grosser *et al.* 2002, Gregory & Jagadeeswawaran 2002, Thattaliyath *et al.* 2005). Thrombocytes present receptors for ATP (Gregory & Jagadeeswawaran 2002) and possibly for other substances, and because they aggregate in a similar way when in the presence of ADP, ATP, collagen, adrenaline, or thrombin (Woodward *et al.* 1981) they are very important physiological substances for vertebrates.

The importance of the response of thrombocytes to both ADP and ATP, in fish, is strengthened by the reported release of the compounds from thrombocytes upon aggregation by adrenaline in concentrations (1.0 μM) that elicit aggregation *in vitro*. The inhibition by adenosine is dose-dependent, suggesting that a common type of action for ADP, ATP, thrombin, and adrenaline generated aggregation of trout thrombocytes (Woodward *et al.* 1981). Activation of fish hemostatic mechanisms following the stress period has also been reported. As the alterations in adrenaline plasma levels are one of the primary effects of stress in fish (Mazeaud *et al.*, 1977, Urbinati & Carneiro 2004), adrenaline plasma levels increase significantly after stress (Mazeaud *et al.* 1977). The facts described above indicate that adrenaline possibly induces the liberation of new thrombocytes into the bloodstream thereby preparing the vascular system for subsequent injury. As a decline of the fibrinogen level occurs after stress (Hatting & Van Pletzen 1997), along with an increase of thrombocytes and the level of adrenaline plasma (Cassilas 1978), there is, consequently, a decrease in the clotting time (Cassilas & Smith 1977). Therefore, this reduction in the time of clotting may be related to an increase in the number of circulating thrombocytes and also of thrombocyte aggregation, when in the presence of adrenaline liberated during stress. Considering that these changes in fish blood coagulation are dramatic, the thrombocytes can be sensitive indicators of stressful situations that are sublethal.

In contrast to the beneficial effects of adrenaline on the clotting system of fish, described above, acute stress might elevate circulating adrenaline levels high enough to cause widespread intravascular clotting and the subsequent death of a fish (Woodward *et al.* 1981). Smith (1980) reports that in *Chanos chanos* Forsskål (1775), *Katsuwonus pelamis* Linnaeus (1758), *Thunnus albacares* Bonnaterre (1788), and *Mugil cephalus* Linnaeus (1758) the spontaneous formation of a thrombus was the cause of death. Presumably, these deleterious effects of adrenaline have also been the cause of the mortality of *O. mykiss* (Bouck & Ball 1966), *C. macropomum* (Gomes *et al.* 2003), and *Perca fluviatilis* Linnaeus, 1758 (Acerete *et al.* 2004) after experiencing different forms of stress. A possible cause of the delayed mortality after fish stress may be intravascular coagulation (Cassilas & Smith, 1977). However, this has not been found to be true by other researchers, which is possibly due to the little attention that has been given to this subject. Although the exact cause of this type of thrombus formation is unknown, it is known that they are constituted by fibrin, thrombocytes, and granulocytes (Lloyd-Evans *et al.* 1994, Hill & Rowley 1996, Hill & Rowley 1998, Hill *et al.* 1999b, Kfoury Jr *et al.* 1999, Jagadeeswaran *et al.* 1999, Gregory *et al.* 2002). The biochemical events leading to thrombosis seem to be the same as those occurring during normal hemostasis, suggesting that dysregulation of this process is critical for the development of this disease (Jagadeeswawaran & Sheeham, 1999). However, the mechanism by which a thrombus is generated is dependent on how the injury is caused (Gregory *et al.* 2002). This mechanism needs to be better understood.

The release reaction in the platelets of mammals is usually preceded by a shape change in which the cells round up, extend pseudopods, and aggregate loosely. At low concentrations of ADP or hydroxytryptamine (5-HT), the platelets do not release their components and subsequently dissociate from each other and return to their normal shape. If the stimulus is great enough, release occurs and irreversibly aggregates the cells. The change in shape allows the platelets to form a firm mass of cells in the site of the vascular injury, which provides the foundation for fibrin deposition and further clot solidification (Harker 1974). According to Woodward et al. (1981), trout (O. *mykiss*) thrombocytes were often aggregated by various aggregating agents, retaining their rod shape, especially when the concentrations of these agents were low. Ultrastructurally, at higher concentrations, there was a tendency for spindle-shaped thrombocytes to be less frequent, and the rounded forms

were predominant. Conspicuous bands of microtubules running throughout the spindle-shaped thrombocytes were also observed, which were possibly maintaining the shape of the thrombocytes until a sufficiently strong stimulus caused a rapid dissociation of the microtubules (resulting in rounded cells). These studies of the ultrastructure of thrombocyte aggregates revealed both forms of thrombocytes, but they usually did not show any precipitated protein that indicated a firm aggregate (Woodward *et al.* 1981), and it was concluded that the reversion from rounded back to spindle-shaped thrombocytes was possible.

It has been suggested that the collection of blood in heparinized tubes, before making blood smears, decreases the percentage of round thrombocytes and increases the percentage of elongate thrombocytes; therefore, a transformation from the elongate form to the round form is involved in the coagulation process (Grizzle & Rogers 1985). On the other hand, recent studies using trout reported that an aggregate formed in the presence of U-46619 and fibrinogen (Lloyd-Evans *et al.* 1994, Lloyd-Evans *et al.* 1994) primarily involved the participation of thrombocytes alone. In the initial response of trout thrombocytes most of the cells in these aggregates displayed extense morphology compared with their normal spindle-shape. Phase-contrast observation of these aggregates also showed that after 12 minutes there were numerous free spindle-shaped thrombocytes, which occurred in *in vivo* unstimulated cells (Hill & Rowley 1996). Similar studies reported about the use of specific monoclonal antibodies (U-46619) analyzed by flow cytometric and immuno-electron microscopy, for trout thrombocytes, and demonstrated that 96% of the characteristic forms of thrombocytes in stimulated or unstimulated fish varied from a round to a spindle form (Kfoury Jr *et al.* 1999). After *in vitro* stimulation using a specific monoclonal antibody (MAb), the morphology of trout thrombocytes, stained with different types of thrombocyte MAbs, changed considerably from their typical oval-shape or spindle-shape form to a more dentritic-like cell morphology, within 24 to 48 hours after the stimulation (Köllner *et al.* 2004). These studies were designed to determine the form of fish thrombocytes during the aggregation process but not the true form of fish thrombocytes when unstimulated (which is discussed below).

Fish thrombocytes are derived from a multipotent type of hemocytoblast capable of differentiation in hematopoietic tissue, such as the spleen, kidney, and liver, depending on the species (Tavares-Dias & Moraes 2004). Usually, in hematopoietic tissue, the thrombopoiesis consists of cells such as pro-thrombocytes, immature pro-thrombocytes, mature pro-thrombocytes, and thrombocytes that are round, oval, and spindle shaped (Esteban *et al.*, 1989). Circulating thrombocytes are variable in shape; they can be round, oval, spindle, fusiform, or spike-shaped cells with long cell processes (Grizzle & Rogers, 1985, Ranzani-Paiva *et al.* 2003, Tavares-Dias & Moraes 2004). These thrombocyte forms seem to be correlated with different stages of maturation. However, young thrombocytes contain a rougher endoplasmatic reticulum and filopodia compared to mature thrombocytes, and they are more rapidly activated (Thattaliyath *et al.* 2005).

Rowley *et al.* (1997) reported that when placed on glass surfaces fish thrombocytes rapidly attach and spread, revealing a variable number of cytoplasmatic vacuoles and a central, often clefted, nucleus. Unlike monocytes and granulocytes, thrombocytes do not appear to move about on glass and they eventually roundup, the nucleus fragments in each cell, and they die. In blood smears of channel catfish an abundance of thrombocyte forms have been described, however round and elongate shapes were predominant (Grizzle & Rogers 1985). A previous review suggest that, due to rapid changes following bleeding, fish thrombocytes exhibit a range of forms namely spiked, spindle, rounded, or fragmented; yet, the spindle morph is probably their true form *in vivo*. Therefore, this variability in thrombocyte structure makes it difficult to identify these cells, and their sometimes rounded appearance in blood smears also causes problems when trying to differentiate them from lymphocytes (Rowley *et al.* 1997). Similarly, we have observed that in several Brazilian fish and exotic species, even when analyzing blood by light microscopy and transmission electron microscopy, the spindle or fusiform shapes have usually been predominant. Moreover, the elongate forms are only occasionally observed, which is probably due to the weak staining of the thrombocyte cytoplasm when using the Romanowsky staining technique. However, elongate thrombocytes are found more easily when the blood smears are stained with alkaline toluidine blue. The spindle morph seems to be the true form of thrombocytes in most live fish. Furthermore, the methods of morphological analysis used and the methods used to analyze the maturation process, in thrombocytes, are responsible for the variety of forms observed. Therefore, assuming that the Woodward hypothesis of a reversible phase of thrombocyte aggregation is true, the spindle shapes are the mature forms while the round shapes are young forms of thrombocytes *in vivo*.

Fish thrombocytes, beyond their primary participation in homeostasis, play an active role in inflammation (Matushima & Mariano 1996, Bozzo *et al.* 2007), as in other non-mammalian vertebrates. It was suggested by Rowley *et al.* (1997) that there is a reversible phase of thrombocyte aggregation that is dependent on the strength of the stimulus. This provides the fish with a sensitive mechanism for responding to vascular injury, so that the unnecessary formation of potentially harmful thrombocyte aggregates is prevented and thrombocytes are conserved when they are not needed. Conserving mature thrombocytes is probably energetically important to the fish (Woodward *et al.* 1981), as fish thrombocytes have both immunological (phagocytic) and hemostatic (aggregatory) functions (Hill & Rowley 1996, Hill &

Rowley 1998, Tavares-Dias *et al.* 2007b), helping to keep the fish free of pathogens following a vascular injury. Therefore, thrombocyte aggregation and phagocytosis, which are mediated by common receptors of thrombocytes, act together on open wounds, through which pathogens could freely pass into, which would be rapidly sealed by the aggregating response of thrombocytes. The first cells that any invading pathogen is likely to come into contact with are thrombocytes, followed by the granulocytes, monocytes, and lymphocytes, which are attracted to the aggregates periphery in the later stages of haemostasis. Interestingly, the platelets of mammals do not have a significantly preponderant role in inflammation (Hill & Rowley 1996, Hill & Rowley 1998, Hill *et al.* 1999) compared to thrombocytes. According to the authors above, the thrombocytes of fish are thought to be the evolutionary forerunners of mammal platelets, and some aspects of the dual functionality observed in fish thrombocytes have been lost during the evolution of the anucleate platelets of mammals, for example, immunological (phagocytic) functions. However, the phagocytic function that occurs in fish thrombocytes has been lost in mammals with the evolution of anucleate platelets. This function seems to have been reduced due to the evolution of leukocytes that are capable of immune response of a greater magnitude, which has not occurred in the leukocytes of fish. Therefore, the leukocytes in fish are able to respond to immunological stimulus, although with reduced magnification compared to mammals.

CONCLUDING REMARKS AND PERSPECTIVES

Fish coagulation mechanisms can be influenced by many factors such as stress, vitamin K-deficiency, liver damage, exposure to chemical products, vascular abnormalities, thrombocyte disorders, and coagulation factor deficiencies. The mechanisms involved in the hemostasis of teleosts are relatively similar to those found in mammals, but they seem simpler. Initially, coagulation begins in few seconds with the formation of a thrombocyte plug in the damaged site and the thrombocytes adhere to the collagen fibers of the blood vessels (tissue factor), using a specific receptor. Consequently, the plasma component responds in a complex cascade to form the fibrin, which reinforces the thrombocyte plug. This process is called secondary hemostasis, which can be divided into the final common pathway, intrinsic pathway, and extrinsic pathway. These pathways are constituted by a series of reactions where a stable form of an activated protein transforms an enzyme that catalyzes the next reaction in the cascade. In teleosts, injury mechanisms involving hemostasis seems relatively simple compared to mammals, because in fish such mechanisms appear to be duplicated.

The thrombocytes, cells that were unknown until the late 1920s to 1930s, play a central role in the coagulation intrinsic system by virtue of their adhesive behavior and also by their ability to aggregate. After vascular damage they adhere to the subendothelial matrix of the blood vessel wall, forming aggregates with other thrombocytes. In this process, the eicosanoids appear to play a significant role because they can stimulate the vasoconstriction or induce local thrombocyte aggregation, or both. The coagulation reaction requires an ordered assembly of enzymes and substrates on a phospholipids surface. Cells can provide an activating surface, but only when phospholipids are present on the external leaflet of the membrane. Thrombocytes are an indispensable source of phospholipids, which activate the coagulation factors, which activates the conversion of prothrombin into thrombin. The spindle morph is the true appearance of the thrombocyte in live fish, and it is the shape of the mature thrombocyte, while the round shape is usually the younger and more active cell morph. Acute stress seems to cause thrombus formation in fish, but the exact cause of this process is still unknown. However, these thrombi are constituted by fibrin, thrombocytes, and granulocytes. Reduction in clotting time during stress can be related to an increased number of circulating thrombocytes and also to a thrombocyte aggregation, when in the presence of adrenaline liberated during stress.

Coagulation factor levels may be used by clinician hematologists to help monitor the health of fish by determining if the level of a factor is low or absent, which is associated with reduced clot formation and bleeding, or too a high level, which is sometimes associated with excessive clot formation. Coagulation factor tests may be used when excessive bleeding is observed, for example, Prothrombin Time (PT) or Partial Thromboplastin Time (PTT). These tests can be used as screening tools to determine if the fish has a coagulation problem. Whole blood clotting time also seems to be a sensitive indicator of the physiological state of fish, and generally when the clotting time decreases the thrombocyte level increases. It is necessary to standardize methods, which should be adapted for fast and reliable analyses of coagulation defects in fish. There is an urgent need to develop accurate methods to count thrombocytes in fish, preferentially, automated methods such as those that are already standardized for counting platelets in species of mammals. In addition, a standardized method of measuring clotting time is of utmost importance, which would allow a comparison among data in the literature. Standardizing how these coagulation parameters are evaluated would contribute to diagnosing animals, with a problem, as fast as possible. In turn this would improve methods of aquaculture because it would facilitate the early detection of blood coagulation problems, which is especially applicable to fish, where the loss of body fluid can be a serious problem.

The impact of the fatty acids on lipid mediator generation also needs to be clarified, because this subject is not well understood in fish. Morphologic and functional studies of the thrombocytes have been reported, but significant questions remain in relation to the regulation

of the coagulation system in live fish. With regard to vasoconstriction in mammals, which is very effective, it is known that thromboxane (TXA_2), prostacyclin (PGI_2), prostaglandin (PGE_2), and the platelet activator factor (PAF) are all involved. Could they also have an influence in the vasoconstriction in teleosts, and in a similar way? In the case of increased membrane lipid content (such as the omega=3 PUFA) the EPA will compete with AA for metabolic action by cyclo- and lipoxygenase pathways? These are worthy and important questions that should be answered in futures studies.

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