A COMPARATIVE STUDY ON THE EFFECTS OF STRESS ON SOME ASPECTS OF *IN VITRO* BLOOD COAGULATION IN TWO FRESHWATER FISH SPECIES.

BY

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DECLARATION

I declare that the dissertation hereby submitted to the University of the North *for the degree* MASTER OF SCIENCE *has not previously been submitted by me for a degree at this or any other university, that it is my own work in design and in execution, except as acknowledged, and that all material contained therein has been duly acknowledged.*

Signed: 

Sello Athlone Rathete

Dedicated to my mother, Salome *and sister,* Johanna *as well as my daughter* Mmanake, *and my son* Rapelo, *for their patience, support and understanding during this study.*

Sello

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ABSTRACT

Clarias gariepinus and ***Schilbe (S) mystus depressirostris*** are generally considered as suitable candidate species for commercial culture in South Africa **(Smit, 1980)**. ***Clarias gariepinus,*** in particular, is popularly used to supplement meat and proteins for workers on large agricultural holdings. This latter species is also the focus of research on artificial propagation by many research institutions in the country. Furthermore, their wide-spread distribution in most of the river systems and large water bodies in South Africa, contributed to their choice as experimental animals for this study.

The emphasis of this study was aimed at evaluating and understanding the possible effects of stress on the blood coagulation system of the two species mentioned. The first problem encountered after sampling blood by cardiac puncture, was the clotting of the blood in ***Clarias gariepinus,*** in spite of the use of sufficient anticoagulant. This probably resulted from the presence of tissue or tissue fluid thromboplastin during collection. Venipuncture was found to be the most convenient and effective means of blood sampling in the two species studied. Thereafter, **Lee -White whole blood clotting times** were determined. These were significantly longer than those for human blood. Furthermore, significant interspecies differences were also recorded for standard, chronically stressed and acutely stressed fish with the latter recording the shortest times in both species.

The different stages involved in blood coagulation were established by thrombelastography. These were found to be similar to those of higher verte-

brates. The effects of the different procedures used for the immobilization of fish in the laboratory, were also evaluated on blood coagulation. Electronarcosis and the use of the chemical anaesthetic, neutralized MS 222, did not produce any detrimental effects. However, the use of natural, acidic MS 222 caused accelerated clotting and can therefore not be recommended for use in fish blood analysis. Chronic and acutely stressed fish produced significant interspecies differences when compared with standard thrombelastograms. These were indicative of accelerated clotting under stress conditions. The cause of such accelerated actions was elucidated by adding artificial stressor hormones to the blood of unstressed fish collected from both species. These results confirmed the role of adrenaline, noradrenaline and cortisol as stressor hormones on accelerated clotting in the two species studied. The addition of higher levels of noradrenaline to the blood, suggested that noradrenaline might accelerate clotting to such an extent, that disseminated intravascular clotting could result in lethal mortalities. The results recorded were therefore indicative of a direct effect on the clotting mechanism as well as an indirect effect through an increase in thrombocyte numbers resulting from contraction of the splenic capsule.

Analysis of the various clotting factors suggested the existence of both intrinsic and extrinsic pathways with clotting factors similar to those of higher vertebrates. However, species specificity in fish brain thromboplastins rendered the use of commercial thromboplastin preparations useless. From all the above-mentioned results, it was concluded that both species had a well developed coagulation system that were possibly stimulated by stress to produce mortalities through disseminated intravascular coagulation. In this re-

gard, it appeared as if ***Schilbe*** was more sensitive to stress stimuli than ***Clarias.*** It also appeared as if both species possessed well-developed fibrinolytic systems to avoid lethal mortalities during the conditions studied.

Thrombocyte aggregatory studies were also undertaken to evaluate the role of these cells during coagulation. Aggregation occurred in a dose dependant manner with the use of **adrenaline, adenosine diphosphate (ADP)** and **arachidonic acid (AHA).** These actions confirmed that fish thrombocyte functions during blood coagulation were similar to those of platelets in human blood, but with significant interspecies differences.

All the above - mentioned results suggested that both fish species responded in a graded manner to the nature and strength of the stress stimuli. It was therefore possible to subdivide stress responses into five different categories, using whole blood clotting times, clotting factor analysis, thrombocyte aggregatory behaviour and thrombelastography as a basis for this purpose. These findings supported the existence of a well-developed clotting mechanism in ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** that were fundamentally similar to those of higher vertebrates.

Finally, the results recorded in this study, provided sufficient evidence for the existence of a general adaptation syndrome **(GAS)** in these two species. This ability to adapt to a changing environment appeared to be different from that of higher vertebrates, probably as a result of differences in the development and anatomy of the central nervous system.

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ABBREVIATIONS

A	=	Adrenaline
ADP	=	Adenosine diphosphate
ATP	=	Adenosine triphosphate
AHA	=	Arachadonic acid
APTT	=	Activated partial thromboplastin time.
BH	=	Benzocaine hydrochloride
Ca^{++}	=	Calcium ions
^{o}C	=	Degree Celsius
C	=	Cortisol
$CaCl_2$	=	Calcium Chloride
DHA	=	Decohexanoate
DIC	=	Disseminated intravascular clotting.
EPA	=	Eicosapentanoate
Fig.	=	Figure
FDP	=	Fibrinogen degradation products
GDP	=	Guanine diphosphate.
K-time	=	Kinetic time
Ma	=	Maximal amplitude
MS 222	=	Methyl sulphonate
MW	=	Molecular weight
NA	=	Noradrenaline.
PF 3	=	Platelet factor 3
PF 4	=	Platelet factor 4

PTT	=	Partial thromboplastin times.
PT	=	Prothrombin times
SEM	=	Scanning Electron Microscopy
TEG	=	Thrombelastogram
TEM	=	Transmission Electron Microscopy
TSC	=	Tri- Sodium Citrate.
r-time	=	Reaction times.

Treatment groups for TEG recordings

A = Neutralized MS 222

B = Electronarcosis

C = Natural MS 222

D = Effects of Natural MS 222 added to blood

E = Neutralized MS 222 without the use of TSC

F = Natural MS 222 without the use of TSC

G = Effects of stress without TSC added

H = Acutely stressed fish

I = Chronically stressed fish

J = Effect of cortisol (C)

K = Effect of adrenaline (A)

L = Effect of noradrenaline (NA)

M = Effect of A and NA

N = Effect of A and C

O = Effect of C and NA

P = Effect of A, C and NA

Q = Effect of A, C and 1\2 NA

CHAPTER 1

INTRODUCTION AND AIMS

CHAPTER 1

INTRODUCTION AND AIMS OF THE STUDY

Physiological research on freshwater fish in the Republic of South Africa (RSA) is a relatively young science, since most of the active research in this field was initiated in the late sixties. However, no local expertise on the artificial propagation of indigenous freshwater fish species in captivity for commercial culture in South Africa was available. Furthermore, rapid progress had to be initiated to promote commercial culture of indigenous freshwater fish. This resulted in **Schoonbee *et al*** (1979) introducing such techniques from the Far East and Israel into the RSA with great success. Such investigations were initially undertaken on an extensive basis in order to provide a reliable and cheap source of animal protein to the lower socio-economic groups in the country. The latter assumption is no longer valid, since it was shown that the major limiting factor for intensive commercial culture of fish was the high cost involved in the preparation and production of commercial feeds for this purpose. In spite of this, such efforts now concentrate on providing economically viable intensive fish production units. These are, however, hindered by many problems of mortalities caused by diseases, transport, handling and the supply of a relatively cheap commercial feed for production purposes. An additional aim of such investigations was to teach and train people in the lower socio-economic groups, some basic principles on fish reproduction and feeding to provide them with sufficient background knowledge to develop their own supply of animal protein to supplement their personal nutritional demands.

Presently, however, a need also exists for overcoming problems associated with mortalities stemming from handling, marking, fin clipping, sorting and artificial spawning in commercial fish culture. These mostly result from stress affecting the various functional systems of fish and consequently, disturbed homeostasis.

It is therefore essential to obtain a better understanding of the underlying mechanisms involved in stress. Clinical haematological and other routine methods for use with human blood, generally served as a basis for providing such information. In many instances, a modification of such techniques was required to produce the desired results **(Blaxhall, 1972)**. The laboratory procedures used for such investigations were evaluated before undertaking such exploratory and informative assignments.

In general, it is agreed that fish exposed to stressful situations are subjected to changes in their homeostatic mechanisms **(Bouck & Ball, 1966; Hattingh *et al* 1975; Smit & Hattingh, 1978)** to ensure their survival under extreme circumstances. In many instances, the functional involvement of many systems cannot accommodate such procedures, and a need therefore also exists, to evaluate the fundamental actions of the various functional systems during stress in order to minimize mortalities. Furthermore, fish researchers were faced with the problem of eliminating or at least minimizing the stressful effects of the various handling procedures. Until recently, the use of chemical anaesthetics, such as tricaine methane sulphonate **(MS 222 - Sandoz)** and acid benzocaine hydrochloride **(BH)**, were widely recommended for immobilizing fish **(Ferreira, *et al* 1979; Smit, 1980)**. Although these

substances anaesthetized the animals effectively and thereby minimized handling stress and other stressful conditions, they also produced side effects on the blood physiology of fish **(McFarland 1959; Smit 1980). Barham *et al* (1987)** reported that, although such side effects might be generally acceptable to researchers in studies on blood coagulation in fish, they could be of sufficient magnitude to mask or significantly influence experimental results. It was also shown that the use of natural **MS 222** as anaesthetic agent resulted in the development of chemical stress **(Wedemeyer, 1970; Houston *et al* 1971 a,b; Sovio *et al* 1977; Smit *et al* 1979)** and that the use of neutralized **MS 222** improved the haematological profiles greatly **(Smit *et al* 1979).** Although sufficient evidence is available on the chemistry, uptake and anaesthetic potency of **MS 222** as well as its effects on blood, the real underlying mechanism of its induction of chemical stress has not yet been elucidated. Nonetheless, due to its relatively high solubility, the use of **MS 222** to immobilize fish, has become so popular, that it is now routinely used for sampling blood for routine laboratory haematological investigations. **Smit *et al* (1979 a)** indicated that anaesthesia with natural **MS 222** caused chemical stress in ***Oreochromis mossambicus*** and ***Cyprinus carpio*** as evidenced from the haematological profiles of these animals. Similar observations were recorded for **MS 222** and benzocaine hydrochloride in rainbow trout, ***Salmo gairdneri,*** by **Soivio *et al (1977).*** **Van Vliet *et al* (1984)** and **Smit & Schoonbee (1988)** suggested that stress may affect the clotting process of fish blood, but no evidence was provided to indicate the possible effects of chemical anaesthesia on blood coagulation in fish. It is therefore essential that the anaesthetic effects on blood coagulation caused by chemical anaesthesia and electro-narcosis be thoroughly evaluated. This in

turn, will help to establish genuine haematological profiles under controlled laboratory conditions.

The effects of stress on blood coagulation and the possible occurrence of **disseminated intravascular clotting** under stress conditions in freshwater fish, have been neglected for a long time. **Doolittle & Surgenor (1962)** and **Lewis (1972)** indicated that the process of blood coagulation in fish is fundamentally similar to that in mammals and other higher vertebrates. Formation of lethal blood clots in fish was also reported by **Smith (1980).** The latter author suggested that these possibly resulted from a variety of factors commonly encountered under aquaculture conditions. Similar deaths of fish caused by spontaneous clot formation have not been reported previously. It was also observed in our laboratory that injury to the gills of certain species, such as ***Cyprinus carpio***, resulted in excessive bleeding and eventual death. It thus appeared that the clotting mechanism in this species may be different to those of higher vertebrates, since the continued bleeding observed, related to a possible bleeding disorder. In addition, it was also observed that blood sampling by cardiac puncture eventually resulted in death, possibly as a result of severing the cardiac conducting system during the sampling of blood. Laboratory experience also suggested that blood sampling by venipuncture should be more effective in terms of fish survival. Such comparative studies have not yet been undertaken. Apart from these observations, it was also noted that fish subjected to acute stress, often displayed white necrotic patches on the skin after a few days. The cause was unknown, but may be due to the formation of tiny clots that block minute blood vessels and thus the subsequent blood supply to certain tissues. No research has thus far been

undertaken to establish the cause of these tiny clots in the circulatory system of fish. It is known, particularly from human medicine, that such thrombi can compromise blood flow sufficiently to cause mortalities. This study will therefore, also look into the possible mechanisms involved in causing this intravascular clotting. **Van Vliet *et al* (1985)** and **Barham (1983)** supported such evidence with the use of thrombelastography, whereas **Fujikata & Ikada (1985)** and **Kawatsu (1986)** also confirmed these observations by applying routine laboratory screening tests. All these observations, however, do not provide sufficient evidence to indicate whether both extrinsic and intrinsic coagulation factors, as identified in mammalian blood, are also involved in the blood coagulation process in freshwater fish.

The laboratory aggregation of human platelets is induced by **Thromboxane A2 (TXA2).** This substance is a metabolic product of arachidonic acid (AHA) **(Hamberg *et al* 1975)**. On the other hand, fish have thrombocytes that are structurally different to the thrombocytes of higher vertebrates. Fish thrombocytes are genuine cells, whereas the thrombocytes in higher vertebrates are fragments derived from megakariocytes **(Archer 1970; Casillas & Smith, 1977; Guyton, 1986)**. Despite such differences, their clotting functions appeared to be similar. It was, therefore, also essential to evaluate the aggregation behavior of fish thrombocytes to identify their role in clotting and their possible effects on coagulation under stress conditions. A thorough evaluation of the effects of stress on blood coagulation will therefore provide suitable information to obtain proper haematological profiles under controlled laboratory conditions, to support proper handling and transport procedures. The above unexplored research areas require detailed

investigations to obtain more information on fish blood coagulation, as well as the possible effects of stress on blood clotting. In this regard the following experimental design was decided upon:

A. EXPERIMENTAL ANIMALS

Clarias gariepinus (sharptooth catfish) and ***Schilbe (S) mystus depressirostris*** (Butter catfish). These species were selected to identify possible interspecies blood coagulation differences and their ability to survive stressful situations. It is also well known that the sharptooth catfish can survive adverse aquatic conditions, whereas butter catfish appears to be more susceptible to stress.

B. BLOOD COAGULATION INVESTIGATIONS CONCENTRATED ON THE FOLLOWING ASPECTS:

1. The use of commercially available polytops for blood sampling and screening tests to establish a low cost basis for blood analysis. It is generally known that the use of non-wettable and vacuum packed tubes, such as siliconized ware, are more expensive. In addition, most of the research was undertaken in the field where non-breakable items were used. It was therefore essential to establish acceptable base line data with the use of these tubes before recording information that could be used for reliable and effective analysis.

2. No information is available on the whole blood clotting times of indigenous

freshwater fish. These were to be compared with values collected for higher vertebrates to identify possible differences.

3. The evaluation of blood coagulation by **thrombelastography** to confirm whether this process is similar to that occurring in higher vertebrates.

4. Determination of intrinsic and extrinsic blood coagulation factors in freshwater fish to establish whether clotting in fish is similar to that of higher vertebrates. The role of these factors before, during and after stress also requires evaluation.

5. The use of commercially available rabbit brain thromboplastin for fish clotting tests and comparison of such results obtained with fish brain thromboplastins. Simultaneously, it is also essential to establish possible interspecies differences with fish thromboplastins.

6. The effects of the chemical anaesthetic, **MS 222 (Sandoz),** in natural and neutralized forms, on blood coagulation in unstressed fish.

7. Evaluation of electronarcosis on blood coagulation.

8. The effects of acute and chronic stress on blood coagulation.

9. Evaluation of stressor hormones on blood coagulation to identify the cause of mortalities resulting from lethal blood clots.

10. The role of thrombocytes in blood coagulation.

It was hoped that the information obtained in this way, might contribute to a better understanding of mortalities resulting form stress.

CHAPTER II

LITERATURE SURVEY

CHAPTER II

LITERATURE SURVEY

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CHAPTER II LITERATURE SURVEY

The general blood coagulation mechanism in fish has thus far been investigated from two standpoints, namely comparative fish physiology and fish pathology **(Kawatsu, 1986). Doolittle & Surgenor (1962)** reported that blood clotting in fish is fundamentally similar to clotting in mammals. **Van Vliet *et al* (1985)** and **Barham (1983)** also supported such results with the use of thrombelastography. **Fujikata & Ikeda (1986)** and **Smit & Schoonbee (1988)** also confirmed these observations by applying routine laboratory screening tests.

Handling stress is an inescapable part of the life of hatchery fishes, whether it occurs during cultural operations or during experimental work in laboratories **(Wedemeyer, 1972)**. However, one stress response that received very little attention in freshwater fish was the effects on the blood coagulation system. **Cassillas & Smith (1977)** reported that the blood clotting mechanism of fish displayed a significant potential of an extremely effective functional responsive system, capable of serving as an indicator of environmental stress. In order to explain the interrelation between the blood clotting process and stress, it is essential to understand the haemostatic process in fish by using the information available for higher vertebrates as a point of departure.

2.1 Haemostasis

Blood maintains tissue stability by keeping the internal environment of the

body constant, so that the normal physiological processes may continue **(Guyton, 1986)**. In order to maintain this stability, blood must remain as a fluid tissue within the confines of the circulatory system. Should an abnormal vascular condition occur, such as an injury to a blood vessel, certain processes are activated in order to stop the flow of blood through the injured vessel wall. Collectively these physiological changes are referred to as haemostasis **(Meyer, 1976; Guyton, 1986)**. Normal haemostasis is dependent on the functions of several physiological mechanisms, whereby, blood maintains its fluidity as well as preventing abnormal loss of blood or injury to the cardiovascular system. Any disturbance in this closely integrated system, may result in abnormal clotting **(thrombosis)** or bleeding, depending on the nature of the abnormality involved. It is not possible to discuss the entire process of haemostasis in this presentation, although an indication has already been provided to outline the purpose of the present investigation. **Haemostasis** generally involves the following processes in higher vertebrates:

- vascular functions
- platelet functions
- fibrinolysis
- inhibitors
- kinins
- compliment activation
- clotting problems

In view of the fact that little information is available on fish haemostasis, the following aspects need to be investigated in greater detail.

(a) a deficiency in or a defect of the coagulation factors,

that may be either congenital or acquired.

(b) the presence in the blood of inhibitors to the action of coagulation factors.

(c) excessive fibrinolysis, platelet disfunction and thrombocytopenia **(Dacie & Lewis, 1984).**

Guyton (1986) explained extravascular, vascular and intravascular mechanisms as the basic facets responsible for haemostasis. These are outlined as follows:

2.1.1 The extravascular mechanism

This mechanism involves the skin, connective tissue and muscle. When these are in abundance at a particular point of injury, the blood escaping from damaged blood vessel, will create a pressure in the surrounding tissue which in turn will eventually cause the blood vessel to be occluded to some extent, especially the smaller veins and capillaries where the blood pressure is relatively low **(Meyer, 1976; Guyton, 1986).** When this occurs, the decreased flow of blood enables the platelets to aggregate more easily at the point of injury. Subsequently, the haemostatic processes that follow are more effective due to the concentration of coagulation factors. Should the blood flow be too rapid, the build up of the necessary haemostatic components is diluted out before becoming effective. However, this mechanism is essentially a physical process and tests to evaluate this response have not yet been devised **(Dacie & Lewis, 1984).**

Limited information on the extravascular response in fish haemostasis is available. It is therefore essential to distinguish between haemostatic responses resulting from open wounds and bruises and intravascular clotting activities.

2.1.2 The vascular mechanism

This mechanism is mainly concerned with the responses of the blood vessels themselves. The efficiency of this process, however, depends on the type of blood vessel involved, ***eg.*** an artery or vein. Also, the age of a particular person should be considered, since blood vessels of older persons tend to be slightly hardened and less elastic due to arteriosclerosis **(Dacie & Lewis, 1984; Biggs, 1976; Meyer, 1976; Guyton, 1986).** According to the latter authors, the vascular mechanism can be described in two separate phases, namely, vasoconstriction and endothelial cleavage. Following injury to the tissue, the local vasoconstriction that follows is probably the most important feature of the whole haemostatic process. The contraction of the injured blood vessel causes a decrease in blood flow, thus enabling the platelets to adhere to the damaged endothelial surface, thereby forming a haemostatic plug. Vascular endothelium has the ability to release a plasminogen activator **(Astrup, 1966).** It was also shown that a tissue factor **(thromboplastin)** is released by the endothelial cells that can possibly also be produced by the monocytes in humans **(Nemerson & Bach, 1982; Dean & Prydz, 1983).** Furthermore, **Becker & Nachman (1973)** also suggested a possible intimate relationship between human platelets and smooth muscle in humans. This observation points to a direct relationship between vascular endothelial cells

and all other components of the haemostatic process. Such functions have not yet been established for fish and do not fall within the scope of this thesis. In addition, many other factors involved in human haemostasis have not yet been identified in fish. Nevertheless, the platelets release a potent vasoconstrictor, **5-hydroxytryptamine,** also known as **serotonin,** which diffuses into the surrounding tissue to cause contraction of the blood vessels in the neighbourhood of damaged tissue **(Biggs, 1976).** Neurological activity due to damage of the smooth muscle layer of the blood vessel (***Tunica media***), also contributes to this constriction, since the smooth muscle contracts as a result of this injury. The combined effects of these two processes result in a spasm of the blood vessel muscle, thereby stemming the flow of blood **(Guyton, 1986).**

The endothelial damage resulting from trauma, causes adhesion of the opposing edges of the epithelial surface, partly due to the constriction effect of the vessel muscle, and also due to the inherent effect of the epithelial tissue, which in some way folds slightly inwards **(Meyer, 1976; Guyton, 1986).** The combined effects of both the extravascular and vascular mechanisms therefore contribute greatly to a reduced blood flow from the site of injury. Vasoconstriction can therefore be regarded as the primary stage of haemostasis, the importance of which should not be underestimated.

Lewis (1972) attempted to determine the effect of vasoconstriction in elasmobranch fish by performing bleeding tests. Small cuts were made in the skin of fish to observe the amount and duration of bleeding. In dogfish, such cuts did not result in bleeding at all. However, results for other species are

not known, but it is assumed that bleeding might have occurred, since the absence of bleeding was only reported for dogfish. Interspecies differences in bleeding times or vascular responses were thus evident. It is therefore essential that these be investigated in future studies.

2.1.3 The intravascular mechanism

The intravascular mechanism is mainly concerned with the changes that occur in the blood at the site of bleeding due to interaction of a number of special proteins, called coagulation factors. The intravascular mechanism can therefore be described in three different stages:

(a) Firstly, the adhesion of platelets at the site of tissue injury and the formation of a platelet plug to decrease blood flow.
(b) Secondly, the platelets release serotonin and a phospholipid factor that result in vasoconstriction and the onset of blood coagulation process, respectively.
(c) Thirdly, the platelets cause retraction of the formed clot to secure the haemostatic plug **(Meyer, 1976; Biggs, 1976; Guyton, 1986).**

2.1.3.1 The blood platelets

During recent years, a virtual explosion of new information concerning the role of human platelets in primary haemostasis and their contribution to intrinsic coagulation and endothelial support as well as their participation in the inflammatory response became available **(Triplett, 1978).** Blood platelets

play a major role in the intravascular clotting mechanism, since they initiate the response immediately after injury **(Meyer, 1976; Guyton, 1986)**. Within seconds of trauma to a blood vessel, platelets adhere to the damaged area, probably due to the exposed subendothelial collagen **(Biggs, 1976)**. Platelet aggregation follows rapidly under the influence of adenosine diphosphate **(ADP)** with the subsequent formation of a primary haemostatic plug **(Born, 1970; Dacie & Lewis, 1984)**. Platelets are also responsible for carrying Factors **I, II, VII, VIII, IX** and **X** that are adsorbed to their surface **(Franz & Coetzee, 1980)**. The importance of these adsorbed coagulation factors lies in the large numbers of platelets aggregating at the site of injury. Such coagulation factors are therefore concentrated at this particular point of injury, thereby enhancing the process of coagulation. During aggregation, platelets release important factors such as platelet factor 3 **(PF 3)** and platelet factor 4 **(PF 4)**. **PF 3** is the phospholipid component of the platelet that is required for the intrinsic coagulation mechanism. This factor is made available only after aggregation has occurred. It will therefore only be found in serum after clot formation **(Meyer, 1976)**. Thus, **PF 3** is also decreased during thrombocytopenia, simply because the platelet number is reduced. However, the quantity of **PF 3** released per platelet may still be normal. No information is available on any possible genetic disorder that effects the quantity of **PF 3** released. According to **Biggs (1976), PF 3** accelerates the interaction between activated **Factors V and X. PF 4** is a low molecular weight, heat stable protein that has anti-heparin activity, either through binding or neutralization. **PF 4** is released in the plasma following aggregation with **ADP, collagen** and **epinephrine.** Increased levels of these compounds are usually found in the plasma following intravascular coagulation, a

condition caused by an increase in platelet destruction as occurs during certain abnormal thrombotic conditions. **Archer (1970)** reported that mammalian platelets differed significantly from fish platelets in this regard.

Fish thrombocytes also play an important role in the initiation of blood coagulation and in clot retraction **(Casillas & Smith, 1977).** It has not yet been established whether all their functions are similar to those of human or other higher vertebrates.

Human thrombocytes are disk shaped with an average longitudinal diameter of approximately 2 - 3 ***μm***. When activated they change from spherical to irregular shapes by shooting out pseudopodia of various lengths. **Woodward *et al* (1981)** indicated that size ranges for fish thrombocytes varied between 10-12 ***μm*** lengths and 4-5 ***μm*** widths. Their sizes were consistent in light, scanning **(SEM)** and transmission electron **(TEM)** micrographs. With light microscopy, control plasma samples contained large numbers of smooth surfaced, rod shaped cells in different freshwater fish species, often containing large indented nuclei. Fish thrombocytes are easily distinguished from other cells when in this form. Polar cytoplasmic extensions or spindles often extend from one or both ends of the rod shaped cell. The **Leishman-Giemsa stain** gave a characteristic pink nucleus and light colored rim of cytoplasm in contrast to the smaller round lymphocytes which had a very dark purple nucleus and a larger light coloured pleimorphic cytoplasm. **SEM** revealed a fairly smooth membrane surface on the rod shaped thrombocyte in rainbow trout whereas aggregates often contained a mixture of rod and round shaped cells. No apparent cross linkages were visible, although large protein

strands, possibly fibrin, were occasionally seen among aggregates. Thrombocytes viewed with **TEM** were recognizable as rods by virtue of a longitudinal section (**Woodward *et al* 1981**). The latter authors also indicated that rainbow trout thrombocytes aggregated in the presence of adenosine diphosphate (**ADP**), adenosine triphosphate (**ATP**), collagen, epinephrine and thrombin. Collagen was the most powerful aggregation compound tested in this species. At a concentration of 100 ***µg/ml,*** collagen produced a 79,5 % aggregation result. The other compounds caused maximum aggregation that ranged from 26 to 43 %. These authors also suggested that adenosine acetylsalicylic acid and prostaglandin E1 inhibited the aggregation of trout thrombocytes induced by **ADP, ATP,** epinephrine or thrombin. On the other hand, collagen caused aggregation of thrombocytes that were not affected by any of the other inhibiting agents. In contrast to thrombin and collagen, **ADP** was never considered as an aggregating agent for thrombocytes of teleost fish and non-mammalian vertebrates (**Lewis, 1972**). This conclusion was premature, since **Woodward *et al* (1981)** observed that **ADP** aggregated trout thrombocytes in a dose dependent manner. Presently, trout thrombocytes and mammalian thrombocytes are the only cells known to aggregate in the presence of **ADP**. Interestingly, **ATP** at identical concentrations (0.5-200 ***µM***) as **ADP**, also strongly aggregated trout thrombocytes. **Stiller *et al* (1974)** reported that bullfrog thrombocytes, that were previously found to be unaffected by **ADP**, were also strongly aggregated by **ATP** (50 -200 ***µM***), but not by guanosine triphosphate (**GTP**). Epinephrine at a concentration greater than 1***µM***, significantly aggregated trout thrombocytes (**Woodward *et al*** 1981). Epinephrine also aggregated mammalian platelets, but no other non-mammalian vertebrate platelets screened, exhibited a similar response. In

fish, as in man, stress elevates circulating epinephrine levels **(Turner & Barnera, 1971; Mazeaud & Mazeaud, 1981).** **Nakano & Tomlinson (1967)** reported plasma epinephrine levels from stressed rainbow trout ranging between 1.0 - 2.0 ***μM***. These were similar to the range for the ***in vitro*** aggregation of trout thrombocytes. In mammals, epinephrine caused the release of new platelets into the bloodstream, thereby preparing the vascular system for subsequent injury **(Ingram *et al* 1977).** Similarly, **Casillas (1978)** reported increased thrombocyte counts associated with elevated plasma epinephrine levels following compression stress in rainbow trout. This supported the earlier findings of **Casillas & Smith (1977),** that the whole blood clotting times of trout blood were significantly reduced following stress. **Woodward *et al* (1981)** presumed that this may be partly due to the increased aggregation of these thrombocytes in the presence of elevated epinephrine levels released during stress. In contrast to these beneficial effects of epinephrine on the fish clotting mechanism, severe stress may elevate circulating epinephrine levels to such an extent, that it may result in wide spread intravascular clotting or disseminated intravascular coagulation **(DIC)** and the subsequent death of fish. Although direct tests were not applied, **Bouck & Ball (1966)** found increased mortalities of rainbow trout following simulated capture and handling stress. This apparently resulted from shock leading to peripheral clotting. These results were supported by **Hattingh & Van Pletzen (1974)** when they investigated the effects of capture and transport stress on fish blood. They reported increased thrombocyte levels in stressed fish. **Casillas & Smith (1977)** recorded similar results during their study on the blood of ***Salmo gairdneri.*** **Kayama *et al* (1987)** studied aggregation responses by the addition of exogenous, highly unsaturated fatty

acids to human platelets and fish thrombocytes. In human blood, aggregation was induced by the addition of arachidonate **(AHA)**, but not by eicosapentanoate **(EPA)** and docosahexanoate **(DHA)**. Furthermore, the addition of **AHA**, produced irreversible aggregation responses in all rainbow trout blood similar to that of human blood. The aggregation of human platelets is induced by **TXA2**, that is derived from **AHA**. The latter plays a major role in human blood coagulation **(Hamberg *et al* 1975)**. **Matsumoto *et al* (1989)** reported that **TXA2** is also a thrombocyte aggregation agent in rainbow trout. These authors also noticed that the mechanism of carp thrombocyte aggregation is different from that of human platelets. Although several reports implicate thrombocytes as clotting cells for fish blood **(Doolittle & Surgenor, 1962; Belamarish *et al* 1966 & 1968)**, few authors qualitatively specified possible thrombocyte functions. Without this information, the understanding of the fish clotting system during stress and other conditions, is not complete.

2.1.3.2 Blood coagulation

The principle of blood clot formation in all species with a circulatory system, has not yet been completely evaluated. On the other hand, this process is remarkable for its efficiency, even though animals that have it are so widely different. As far as vertebrates are concerned, the greatest amount of research has been undertaken on human subjects, but is not yet complete **(Archer, 1970; Guyton, 1986)**. In man, blood coagulates as a result of a series of proenzyme to enzyme changes that are described in the form of a series of **cascade reactions (Guyton, 1986)**. Human plasma contains

approximately a dozen protein factors that are concerned with blood coagulation. These factors are designated by roman numericals according to the recommendations of the **International Committee** responsible for standardizing blood nomenclature.

The essential reaction in coagulation is the conversion of the soluble protein **fibrinogen** into insoluble **fibrin** by means of an enzyme known as **thrombin. Fibrinogen** is present in the circulating blood, whereas thrombin is formed from an inactive circulating precursor, known as **prothrombin,** when bleeding occurs. The activation of thrombin depends on the presence of calcium **(Ca)** ions and certain factors that are derived from the damaged tissues as well as disintegrated platelets and from the plasma itself. The reactions following stimulation of these factors, result in coagulation of blood occurring by two major pathways. These are described as the **intrinsic** and **extrinsic** mechanisms of blood coagulation **(Keele, 1982)**. Factors **XII, XI, VIII, X** and **V** and **prothrombin** participate in the intrinsic pathway whereas tissue factor, generally known as **thromboplastin,** participates with factors **VII, X, V** and **prothrombin** in the extrinsic pathway to initiate clotting.

The extrinsic pathway

The extrinsic pathway is usually activated by injury to the tissue. When tissue cells are damaged as a result of trauma, a series of reactions occur to initiate clotting. The first phase involves the release of a tissue extract, known as **factor III.** The latter provides essential phospholipid components that, in conjunction with **factor VIII** and **ionized calcium,** activate **Factor X** to

activated factor X. Thereafter, tissue **thromboplastin** converts **prothrombin (factor II)** into **thrombin.** The **thrombin** in turn converts **fibrinogen** into **fibrin** as illustrated in **Fig. 1. Thrombin** also acts on **Factors V** and **VIII** as a surface active catalyst. It also has a so-called release reaction effect on the platelets whereby active substances, such as **PF 3,** are released from platelets to support the clotting process **(Biggs, 1976).**

Doolittle and Surgenor (1962) investigated some aspects of extrinsic clotting in fish. They discovered that plasma clotting was greatly accelerated by the addition of homogeneous tissue extracts. Relative species specificity was also established. In his studies on freshwater fish, **Van Vliet (1981)** recorded higher prothrombin times with the use of standard rabbit brain thromboplastin when compared with results obtained from human blood. **Lewis (1972)** also investigated the effects of tissue extracts on fish blood coagulation, using the skate as an example. It was found that clotting did not occur, unless calcium was added to the plasma - tissue extract mixture. Nevertheless, calcium, in normal physiological concentrations, gave good clotting reactions in teleost fish, but lamprey plasma did not clot with any concentration of calcium. **Doolittle and Surgenor (1962)** investigated this phenomenon and suggested that the high concentrations of urea and trimethylamine in elasmobranch blood, resulted in an altered calcium activity or abnormal binding. Further studies, however, proved that this was not the case. These found that elevated concentrations of calcium produced mild inhibition of clotting in the extrinsic pathway of the dogfish and lamprey eel. The final conclusion made was that calcium, in various concentrations, is essential in both the extrinsic and intrinsic clotting pathways. **Van Vliet (1981)** recorded shorter

prothrombin times when using fish brain thromboplastin. All these findings, however, did not provide sufficient evidence to indicate clearly which clotting factors are involved and whether the clotting process includes the same basic processes as those in mammalian blood.

The intrinsic pathway

The intrinsic clotting pathway involves only blood plasma clotting factors. Although thrombin can initiate action in platelets, other substances also seem to display this ability. These include **collagen, elastin** and other connective tissue elements. The interaction of these factors is known to be complex. The precise mechanism of clotting in the organism is still not fully elucidated **(Wilson, 1979)**. Basically, two events occur very soon after trauma. Firstly, platelets start to adhere and aggregate at the site of bleeding. Secondly, **factor XII** is activated due to surface contact with the damaged tissue and converted into **activated factor XII (XIIa)**. This triggers off the so-called cascade sequence of coagulation reactions as illustrated in **Fig. 1 (Biggs, 1976)**. **Activated factor XII** acts on **factor XI** and produce **XIa**. This factor, in turn, changes **factor IX** into **IXa,** which again activates **factor VIII** into **VIIIa. VIIIa** acts on **factor X** to produce **Xa** which, together with **Factor V,** converts **prothrombin (factor II)** into **thrombin (IIa)** in the presence of calcium **(factor V)**. Finally, thrombin acts on **factor I (fibrinogen),** which is changed into a fibrin monomer **(Ia)**. **Factor XIII,** in the presence of **factor VIII** and **calcium,** polymerized the fibrinogen into a stable fibrin polymer **(Dacie & Lewis, 1984; Meyer, 1976; Biggs, 1976)**.

It should be noted that both extrinsic and intrinsic pathways use **factors X** and **V** to form **thromboplastin.** This substance has the ability to convert prothrombin into thrombin. However, each of these pathways eventually produce thromboplastin in its own way. The main difference between the two pathways lies in the activating mechanism. Both pathways also require **factors X** and **V** in the final coagulation steps. During normal physiological coagulation, these reactions occur simultaneously at the point of injury **(Biggs, 1976).** The fibrin strands thus formed, act as a network which entraps the cellular components of the blood, thereby producing a haemostatic plug **(Wilson, 1979).**

Little information is available on the intrinsic pathway of fish blood. **Doolittle & Surgenor (1962)** investigated prothrombin activity in the blood coagulation system of cyclostome, elasmobranch and teleost fish and found that the plasma of all three classes contained a factor, presumably **prothrombin,** that is essential for clotting. **Lewis (1972)** also investigated the intrinsic pathway in elasmobranch blood and recorded extremely long clotting times, some up to 48 hours, similar to those obtained by **Doolittle & Surgenor (1962). Kawatsu (1985),** in turn, established standard procedures for the determination of clotting times in order to detect the factors associated with certain human disorders in the haemostatic system of common carp. The latter author observed differences in calcification times, prothrombin times **(PT),** partial thromboplastin times **(PTT)** and activated partial thromboplastin times **(APTT),** thereby suggesting that a well functioning intrinsic haemostatic system is available in carp blood. It was also found that citrated plasma lost its clotting activity within 24 hours during incubation at 37 °C. On the other

hand, clotting activity was maintained for a longer time at 25 °C. The shortest clotting times were obtained with a mixture of barium sulfate ($BaSO_4$) adsorbed plasma serum. This suggested that the coagulation factors in carp blood can be classified into serum and barium sulfate factors.

Van Vliet (1981) reported that prothrombin times obtained with human and fish blood showed significant differences due to species specificity. When using commercially available rabbit brain thromboplastin for human blood, prothrombin times where higher in fish when compared with human blood. Prothrombin times recorded with laboratory prepared thromboplastins of ***Oreochromis mossambicus*** and ***Cyprinus carpio,*** produced no significant clotting differences with human blood at 20°C. However, carp plasma and thromboplastin prepared from both species, resulted in reduced prothrombin times at 37 °C. This suggests a possible temperature liability of carp plasma, thereby indicating a possible adaptability to lower temperatures (10 - 30°C) when compared with ***O. mossambicus*** (15 - 40 °C). Although blood clotting in fish appeared to be fundamentally similar to that in mammals, no accurate assays for these intrinsic factors have yet been reported. Valid assays for these factors are important for comparative interspecies fish investigations and for comparison with human blood. Furthermore, no information regarding the effects of stress on factor activity is available. It is essential to investigate this aspect, since it is generally known that stress affects the haematological profiles of freshwater fish.

Clot retraction

Once the fibrin has formed and the opening in the blood vessel has been sealed off, more fibrin threads are deposited over and around the site of injury, thereby anchoring the plug firmly to the vessel wall. Furthermore, polymerization of the fibrin strands now takes place under the influence of platelet factors **(Biggs, 1976; Meyer, 1976)**. The formed clot now starts to retract, thereby drawing the walls of the wounded tissue together. This may promote haemostasis, since retraction of the thrombus could prevent total occlusion of the blood vessel **(Meyer, 1976)**. However, the activity of fibroblasts at the site of injury permanently seals the damaged tissue by forming a permanent connective tissue plug. However, limited information is available on this aspect in fish and should be further investigated in the near future **(Van Vliet, 1981)**.

The fibrinolytic system

As blood clots, fibrin is laid down as a network of fine threads which entangles the blood cells. The freshly formed threads are extremely adhesive, since they stick to each other, the blood cells, the tissues and to certain surfaces **(Keele, 1982)**. The dissolution of a clot is a cascade process similar to clot formation. The fibrinolytic system is composed of an inactive precursor, activators and inhibitors. The two main functions attributed to the fibrinolytic system during its normal response to injury, include restriction of fibrin formation to the area of injury and clot dissolution through enzymatic digestion of the formed fibrin **(Britton, 1963; Dacie & Lewis, 1984; Keele,**

1982).

When both the clotting and the fibrinolytic systems are in balance, a normal response to injury occurs. Fibrin is formed, tissue is repaired and fibrin is gradually digested. During this normal response, the process of fibrin formation and fibrin dissolution proceed simultaneously, the latter however, at a slower rate. The initiation of fibrinolysis is not totally independent from the body's normal responses occurring after injury. However, upon activation, the term primary fibrinolysis is used to identify the first fibrinolytic reaction. Such initiation reactions occur when previously inert blood activators, tissue cells, secretions and vascular endothelium, are released into the peripheral circulation in their active form. These activators rapidly convert plasminogen to plasmin, resulting not only in the consumption of **factors I, V** and **VIII,** but also in the degradation of the circulating fibrinogen and **factors V** and **VIII,** with subsequent fibrin or fibrinogen degradation products **(FDP).**

Physiological stimuli that initiate primary fibrinolysis, include strenuous physical exercise, emotional stress, severe anxiety and anoxia **(Sherry *et al* 1959; Dacie & Lewis, 1984; Meyer, 1976).** The active enzyme, plasmin, has the ability to digest both fibrin and circulating fibrinogen. In both instances, **FDP** are formed.

Two main types of **FDP** are recognized. These include small fragments that are not clearly defined, and larger, higher molecular weight fragments that undergo several intermediate changes and are finally degraded to a core. The latter is resistant to further action of plasmin. This has the ability to form a

clot upon the addition of thrombin, although the time required for clotting is prolonged. Fragments **Y, D** and **E** are smaller and are considered to be inhibitors of clot formation **(Marder & Shulman, 1969; Meyer, 1976).**

Hougie (1971) observed poor clot retraction and serum haemolysis in both sick and healthy pacific salmon. This suggested that there was no lysis of any of the whole blood clots formed. Although **Doolittle & Surgenor (1962)** tried to describe clot lysis in fish blood, no intensive investigation into this aspect of blood coagulation has been undertaken. Future investigations should therefore look into the role of stress on the fibrinolytic system.

Thrombosis

Mackie and Douglas (1977) described the main factors in the pathogenesis of thrombosis as being injury to the blood vessel wall and stasis within the lumen of the vessel, provided these are associated with platelet aggregation or activation of the clotting factors in the blood. The resolution of small thrombi and fibrin deposits in the blood vessel must be considered as a regular process occurring in the organs, since constant minor injuries occur regularly during stressful situations that are experienced daily. This results in the release of thromboplastin agents from the tissues **(Astrup, 1956).**

Smith (1980) provided evidence for spontaneous thrombin formation in live fish. He observed the sudden and unexpected death of an adult milkfish. The ***post mortem*** investigation revealed a thrombus occluding the lumen between the bulbous arteriosis and the ventricle. The formed thrombus appeared to

be closely contoured to the heart wall. It was homogeneous in appearance and showed a layering of fibrin, thrombocytes and blood cells. This differed from the soft gelatinous ***post mortem*** clot usually found after death. Other similar thrombi were also detected in this fish. Available literature on thrombin formation within living fish is extremely limited when compared with similar reports for mammals. It does, however, appear that similarities between fish and mammalian coagulation systems are possible. It was therefore suggested that this phenomenon may also occur in fish blood **(Doolittle & Surgenor, 1962; Lewis, 1972).**

Irani & Cliffton (1970) investigated the effects of haemorrhagic shock on blood coagulation and metabolic changes in dogs. These authors concluded that such changes coincided with severe or irreversible shock and were extremely complex and very difficult to evaluate. However, their results support the opinion that hypercoagulability and intravascular coagulation occur during haemorrhagic shock. The same pattern of increased coagulability was also observed in fish **(Bouck & Ball, 1966; Mazeaud *et al* 1977; Casillas & Smith, 1977; Woodward *et al* 1979).**

3. Thrombelastography

Thrombelastograghy allows a continuous visual kymographic observation of a blood or plasma sample during all phases of coagulation **(Fig. 2).** The resultant recording obtained, is known as a thrombelastogram **(TEG - see Figure 3).** The **r-time** is equivalent to thromboplastin generation and represents the factors that take part in the intrinsic pathway of coagulation.

The **k-time,** also known as the thrombin constant, measures the speed at which a clot of certain solidity is formed. **The sum of the r and k values** represent the kinetic coagulation phase. The latter is equivalent to the total coagulation time of the sample. The **maximal amplitude (Ma)** represents the solidity that a given clot has attained and thus represents the dynamic properties of the formed fibrin, platelet action and calcium **(De Nicola, 1957; Van Vliet, 1981).** The latter author also suggested that thrombelastography can be effectively used as a diagnostic tool in fish research, since blood coagulation in fish appeared to be fundamentally similar to that of higher vertebrates, involving the same basic processes ***ie.*** extrinsic and intrinsic thromboplastin generation, the conversion of prothrombin to thrombin as well as the formation of fibrin from fibrinogen. This evidence was also supported by **Barham (1983)** using thrombelastography. These findings suggest that thrombelastography can be effectively used to demonstrate stress effects on blood coagulation in fish. The results obtained suggested that hypercoagulability was evident under stress conditions. Such observations supported the findings of **Casillas & Smith (1977),** who found that the blood clotting times declined after stress in both strains of rainbow trout, ***Salmo gairdneri.*** All these findings, however, did not clearly explain the factors associated with stress related clotting changes. It is also clear that further investigations have to be undertaken to describe the haemostatic process in freshwater fish in greater detail.

4. Anaesthetics

The use of chemical anaesthetics is an everyday practice in fish culture. **Bell,**

(1967) compiled most of the relevant papers on the use of these chemicals in fish. Intensive culture of fish often necessitates the use of various handling and transport procedures, that ultimately cause stressful reactions. These usually result in a high mortality rate in certain fish species due to possible osmoregulatory disturbances **(Hattingh, 1976)**. In general, research on the effects of stressors in fish, attempt to eliminate or at least minimize the stressful effects of the handling procedures inherent to such investigations.

It is well known that handling and anaesthesia induce changes in the physiological status of fish **(Wedemeyer, 1970; Soivio & Oikari, 1975)**. It is also reported that the use of the chemical anaesthetics, **benzocaine hydrochloride** and **MS 222 (Sandoz)** in their natural forms, affect the haematological profiles of freshwater fish **(Smit *et al* 1979b; Ferreira *et al* 1981). Smit *et al* (1979 a)** also reported that chemical anaesthesia with **natural MS 222 (Sandoz)**, resulted in chemical stress as evidenced by the blood parameters of the anaesthetized fish. Similar observations were also recorded by **Sovio *et al* (1977)** for **MS 222** and **benzocaine** in rainbow trout, ***Salmo gairdneri.* Smit *et al* (1979 a)** have also indicated that such changes in the haematological profiles induced by the natural anaesthetic, can be avoided by the use of the neutralized compound. Since these compounds are generally expensive, **Barham *et al* (1987)** investigated the effects of electronarcosis as a possible suitable alternative to chemical anaesthesia. His findings on haematological profiles suggested that these were less affected by this means of immobilization when compared to chemical anaesthesia. In addition, this immobilization procedure was also more cost-effective. A few studies to date also suggest that recovery is uneventful and possibly also less

stressful **(Kynard & Lonsdale, 1975; Madden & Houston, 1976)**. However, the possible effects of immobilization by electronarcosis on blood coagulation, are not yet known. This aspect therefore requires more detailed investigations. This is in contrast to the information available for chemical anaesthesia, that explains satisfactorily the haematological changes associated with the use of natural MS 222. Furthermore, no comparative information on the effects of chemical anaesthesia and electronarcosis on blood coagulation is available. These need to be effectively evaluated in order to determine their suitability for fish blood coagulation diagnostics.

5. STRESS

The concept of biological stress is a complicated phenomenon to which there is no simple definition. It appears to affect all functional systems, of which there are at least eleven, depending on the manner of classification **(Pickering, 1981)**. In addition, the severity of the responses by different functional systems, depends on the degree of exposure to stress. It is therefore essential to obtain a better understanding of the fundamental principles involved in stress in order to evaluate the degree and nature of any stress reaction. Such reactions consist of an immediate response in order to adjust to a specific situation, a longterm adjustment to cope with the changed environment followed by a return to normality. These responses will be discussed in greater detail in **Chapter 6 (Discussion)**. The most important aspect about stress, is that such responses can be measured. Such measurements therefore provide an indication of the adaptability of a particular fish species to maintain homeostasis. Such effects are of significant

economic significance, since some species are more susceptible to a changing environment than others. In general, however, most processes involved in stress still remain poorly understood.

Mazeaud *et al* (1977) and **Schreck, (1981)** reported that acute forms of handling stress associated with routine hatchery procedures, almost invariably induce marked physiological stress responses characterized by elevated levels of plasma catecholamines and corticosteroids. **Mazeaud *et al* (1977)** found that stress elevated catecholamine and cortisol levels in freshwater fish, but no acceptable explanation was provided for such possible primary hormone effects on blood coagulation in particular. It is therefore essential to consider and investigate the possible effects of stress on the normal clotting procedures, and to determine whether the addition of stressor hormones to the normal blood of unstressed fish would invoke the clotting changes observed in stressed fish.

CHAPTER III

MATERIALS AND METHODS

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Adult healthy specimens of both sexes of ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** were collected from **Mokgoma Matlala Dam** near **Marble Hall (Transvaal, S.A).** The fish were transported in well-aerated fresh water and acclimatized for at least three months in the aquarium at a temperature of 22 ± 1°C. On arrival at the laboratory, the aquarium was treated with a zinc-free malachite green-formalin mixture for four hours to minimize fungal infection **(Conroy & Herman, 1970; Smit, 1980; Van Vliet, 1981).** During this period the fish were fed commercial protein rich trout pellets supplied by Trukka **(Van Vliet, 1981).** Two weeks before the start of the experiment, the fish were transferred to smaller laboratory aquaria to become accustomed to laboratory conditions. From this time on, they received no food. For a possible further reduction of stress effects, individual fish were kept in smaller aquaria of 60 liter capacity for twenty four hours prior to use **(Van Vliet, 1981).**

3.2 Blood coagulation studies

Blood coagulation was evaluated with a **HELLIGE THROMBELASTOGRAPH D.** According to the manufacturer's instruction manual, this technique is very sensitive to personal and environmental effects in producing artifacts.

3.2.1 Stock solutions of Tri-sodium citrate (TSC) and calcium chloride.

A 3.8 % stock solution was prepared so that small amounts could be used with each blood sample. In this way, the dilution factor could be minimized. Also, easily measurable amounts of anticoagulant could be delivered into the test tubes with the aid of micro pipettes. To determine the most effective concentration of **TSC** for obtaining a reliable standard thrombelastogram (giving the so-called wine glass shape with fish blood), a stock solution of **TSC** was prepared by dissolving 3.8 grams Tri-Sodium Citrate in 100 ml double distilled water **(Van Vliet 1981).** A 1.29 % solution of anhydrous $CaCl_2$ was prepared as recalcifying solution.

3.2.2. Blood sampling for thrombelastography.

Care was taken that the fish for these experiments, were not disturbed to minimize possible stress effects. Blood sampling was performed with utmost care to limit contamination of blood with tissue fluid. Only blood samples obtained with direct venipuncture were used. All other samples were discarded, since the slightest negative pressure on the syringe would allow tissue fluid or body fluid to enter the syringe, thereby initiating the blood to clot. **Van Vliet (1981)** termed the "clean" blood samples as "atraumatic samples". **Neutralized MS 222, Natural MS 222** and **electro-narcosis** were used to sedate the fish **(Ferreira *et al* 1979; Smit *et al* 1980; Barham *et al*** 1989). Needles (23 g x 1,5) and plastic disposable syringes were used to obtain blood by direct venipuncture. Blood and anticoagulant was mixed in the ratio of 1 part **TSC** to 9 parts of blood. This resulted in a final

concentration of 0.1 mg **TSC/ml** blood. The blood and anticoagulant were mixed carefully and stored at room temperature until the tests commenced. In this study, the average time lapse before **TEG** evaluation started, was 10 minutes. The sample size was 10 fish per determination.

3.2.3 Thrombelastographic Studies

All tests were done at 37°C on a **Hellige Thrombelastogram D.** Attempts to disconnect the heater element, were not successful. The instrument was switched on at least ten minutes prior to use. Cleaning of the piston and cuvettes was carried out according to the instructions of the manufacturer and great care was taken to remove all residual fibrin and plasma before introducing any sample. All components were handled with fine tissue paper to avoid finger contact, since any dirt or scratches could greatly modify the results. The cuvettes were placed in position and allowed to warm up for ten minutes. Thereafter the piston was placed in position and the chart drive switched on. The blood sample was carefully mixed by inversion. Blood (250 **μl)** was transferred to the cuvette with a micro pipette, followed by 100 **μl** of 1.29 % $CaCl_2$ solution. Care was taken to prevent foaming. The piston was lowered carefully into the cuvette and the surface of the blood covered with a thin layer of liquid paraffin to prevent drying out of the sample. Each thrombelastogram tracing was carried out for 30 minutes, after which the reaction time **(r),** kinetic time **(k)** and the maximum amplitude **(Ma)** were measured. The index of thrombodynamic potential **(ITP)** was then calculated. Individuals of both fish species used in this investigation were subjected to the following treatments in order to evaluate their effects on blood

coagulation.

3.2.3.1 Effects of anaesthetics

3.2.3.1.1 Neutralized MS 222 anaesthetized fish

Ten fish of both species with approximately equal mass, were put into **60 l** well-aerated aquaria for **24** hours prior to use. Thereafter the fish were anaesthetized with **120 mg/l of neutralized MS 222 (Smit, 1980).** Blood was collected atraumatically by means of venipuncture and **TEG** patterns were subsequently recorded. Atraumatic blood samples and thrombelastogram patterns were obtained as previously described.

3.2.3.1.2 Electronarcotized fish

A unit for **A C Narcosis**, similar to that designed by **Barham *et al* (1987)**, was constructed in the laboratory. The electronarcosis unit was attached to stainless steel electrodes that totally covered the ends of the aquarium tanks in order to ensure parallel lines of force in the electrical field **(Barham *et al* 1987).** Experimental groups consisted of 10 fish for each species. To evaluate the narcotizing effects on blood coagulation, acclimatized fish were removed from holding tanks and placed in 60 liter well aerated aquaria fitted with aluminium electrodes. Current was applied for 30 seconds after which the fish were removed easily from the water and subjected to a clean venipuncture. Thrombelastogram patterns were obtained as previously explained.

3.2.3.1.3 Natural MS 222 (Sandoz) anaesthetized fish

In order to minimize possible effects of handling stress, individual fish were transferred to smaller aerated 60 liter aquaria twenty four hours prior to use. **MS 222** was used at a concentration of 120 mg/l in its natural acidic form **(Smit *et al* 1979a)**. After complete sedation, fish were removed gently from the tanks and blood samples collected by direct venipuncture. Thrombelastogram patterns were recorded as previously explained.

3.2.3.1.4 Neutralized MS 222 anaesthetized fish without TSC

The ratio of anticoagulant and blood is of the utmost importance, since excessive amounts of anticoagulant will certainly influence the results of almost any test carried out **(Smit & Schoonbee, 1976)**. In this experiment it was essential to observe whether the absence of anticoagulant will change the shape of the **TEG** patterns recorded. Ten fish of both species were transferred to smaller aquaria after which they were anaesthetized with **neutralized MS 222 (120 mg/l)**. Blood was sampled atraumatically and no **TSC** was used to record **TEG** patterns.

3.2.3.1.5 Natural MS 222 anaesthetized fish without anticoagulant.

The same procedures explained in **3.2.3.1.4** were repeated, but in this case **MS 222** was used in its natural form.

3.2.3.1.6 MS 222 added to the blood

It was also essential to evaluate the direct effect of acidic **MS 222** on blood coagulation. Acclimatized fish of both species were transferred to smaller aquaria, and thereafter blood samples were collected by venipuncture. **TSC** (0.1ml) was carefully mixed with 0.9 ml of blood. Thereafter, **MS 222** (0.01g) was added to the citrated blood. **TEG** patterns were recorded as explained previously.

3.2.3.2 Unanesthetized fish

During this procedure, fish were removed from smaller aquaria, held firmly to avoid struggling and the blood sampled immediately. Blood was then carefully mixed with **TSC** and thereafter the necessary **TEG** patterns recorded.

3.2.3.3 The effects of stress on blood coagulation

Different types of stress that fish could possibly experience, were used to evaluate effects of stress on coagulation. The types of stress imposed on the experimental animals were as follows:

3.2.3.3.1 Acutely stressed fish

Ten fish of both species were transferred to individual smaller aquaria of 60

liter capacity for 24 hours prior to use. Thereafter, they were removed from the tanks and placed on the floor for 5-10 minutes. The fish were jumping up and down until they came to rest. No anaesthetic was used to collect blood samples. Blood was collected atraumatically by holding the fish firmly during sampling to avoid struggling. The additional stress caused by firmly holding the fish, would probably be responsible for only a small fraction of what the fish really experienced during the induced stress. **TEG** patterns were recorded as explained previously.

3.2.3.3.2 Chronically stressed fish

Ten fish of both species were transferred to individual smaller aquaria of 60 liter capacity for 24 hours prior to use. The water level was dropped to 6 liters to just cover the fish. All fish were allowed to splash for several minutes (30 min) prior to sampling. Blood sampling was again performed as described in the previous section for acutely stressed fish without the use of an anaesthetic. **TEG** patterns were recorded as explained previously.

3.2.3.4 The effects of adding artificial stressor hormones to evaluate blood coagulation.

In order to explain accelerated clotting during stress in fish, it was necessary to evaluate the effects of stressor hormones on blood coagulation by adding small quantities of such hormones to the blood. Cortisol **(MW = 360.5),** adrenaline **(MW = 183,2)** and noradrenaline **(MW = 169,2),** obtained from the **SIGMA CHEMICAL COMPANY,** were used in various combinations.

During this experiment, care was taken to minimize handling stress and other stressful situations. Neutralized **MS 222 (Sandoz - 120 mg/l)** was added to the aquarium water in such a way that the fish did not realize what they experienced. Blood was sampled atraumatically and then mixed carefully with the **TSC** (0.1mg/1 ml blood). Thereafter, the different artificial stressor hormones were added in the following combinations:

(a) Unstressed fish with 0.1mg of cortisol **(C)** added to 1 ml blood.
(b) Unstressed fish with 0.1 mg of adrenaline **(A)** added to 1 ml blood .
(c) Unstressed fish with 0.1 mg of noradrenaline **(NA)** added to the blood.
(d) Unstressed fish with 0.1 mg each of **NA** and **A** added to blood.
(e) Unstressed fish with 0.1mg each of **NA** and **C** added to blood.
(f) Unstressed fish with 0.1 mg each of **A** and **C** added to 1 ml of blood.
(g) Unstressed fish with 0.1 mg of **A, C** and **NA** added to 1 ml of blood.
(h) Unstressed fish with 0.1 mg each of **C** and **A** and 0,05 g of **NA** added to 1 ml of blood.

TEG patterns were obtained for each treatment group as explained previously.

3.3 Whole blood coagulation time tests

A method based on that of **Lee and White (Dacie & Lewis, 1984)** was adopted to determine the coagulation time of whole blood clotting time for both stressed and unstressed fish. In both instances, ten fish of each species were anaesthetized with neutralized **MS 222 (Sandoz)**. Two ml of blood were

sampled atraumatically by venipuncture with a plastic syringe as explained previously. Immediately thereafter, 1 ml of blood was transferred into each of two polytop test tubes. These tubes were slowly tilted until the blood became visibly viscous and ceased to flow freely. At this point, the stopwatches were stopped **(Van Vliet 1981)**. The coagulation times per fish were recorded as the average obtained from both polytops. Fish were acutely stressed as explained in **3.2.3.3.1** and similar procedures described above, were followed.

3.4 Clotting factor determinations

Coagulation factors for both intrinsic and extrinsic clotting pathways were determined with the aid of **Boehringer Mannheim Test Combination Kits.** Fish were anaesthetized with **neutralized MS 222 (Sandoz 120 mg/l)** as described by **Smit *et al* (1979a)**. Atraumatic blood samples were obtained from both species and mixed carefully with tri-sodium citrate to ensure a concentration of **0.1 mg TSC per 1 ml of blood.** Immediately thereafter, the blood was centrifuged at 200 g for 15 minutes. The plasma was transferred into clean polytop tubes and stored at room temperature prior to use. The following factors and their activities were determined according to the procedures outlined for each test kit:

(a) Factor II **(Cat. no. 126 667)**

(b) Factor V **(Cat. no. 126 675)**

(c) Factor VII **(Cat. no. 126 658)**

(d) Factor X **(Cat. no. 126 683)**

(e) Factor VIII **(Cat. no. 612 683)**

(f) Factor IX **(Cat. no. 612 111)**

(g) Factor XI **(Cat. no. 612 154)**

(h) Factor XII **(Cat. no. 612 138)**

All test were completed at room temperature.

3.5 Preparation of fish brain thromboplastins

Fish brain thromboplastins were prepared as described by **Dacie & Lewis (1984).** These were used to determine **PT times** and compared with those used for diagnostic aids in clinical medicine.

3.6 Aggregation studies

Since differences exist between mammalian platelets and fish thrombocytes **(Archer, 1970, Cassilas & Smith, 1977),** it is not possible to adopt the same aggregation study procedure employed with human blood for that of fish blood. For this reason it was decided to use some of the less complicated tests available in order to investigate aggregation behavior of fish thrombocytes.

3.6.1. Aggregation behavior of fish thrombocytes

Aggregation of fish thrombocytes was studied by an electrical impedance method employing the whole blood electronic aggregometer **(CHRONO-LOG**

530) equipped with a suitable pen recorder. Atraumatic blood samples were collected from 10 anaesthetized **(Neutralized MS 222)** fish of both species by using tri-sodium citrate as an anticoagulant in the ratio of 1:9. Aggregation was determined in a cuvette stirred at 25°C. Effects on thrombocyte aggregation were determined by adding **arachidonic acid (1 mM), adenosine diphosphate (1 mM) and decosahexaenoic acid (1 mM)** in increasing concentrations (2,5 μl; 5 μl; 10 μl and 15 μl) to the samples.

4. Statistical analysis

Statistical computations were undertaken with an **OLIVETTI** Computer employing **SAS** programmes. All differences were considered as significant at the 5% level ($p < 0.05$). Correlation coefficients **(r)** were regarded as highly significant for values above 0.90 and as significant for values between 0.80 and 0.89. Values ranging from 0.70 to 0.79 were taken as meaningful whereas those between 0.50 and 0.69 as indicative.

CHAPTER IV

RESULTS

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CHAPTER IV RESULTS

Although routine coagulation profiles usually include the determination of plasminogen, thrombin clotting times, fibrinogen levels, platelet counts, etc, the **TEG** appears to provide a simple and rapid additional means to evaluate the therapeutic application of a highly complex medical condition concerning coagulation in human blood. It was, however, indicated in **Chapter 2**, that thrombelastography can also be used as a diagnostic tool for assessing stress and to determine intra and interspecies differences in freshwater fish.

The aim of this study was to evaluate the effects of stress on blood coagulation with the use of the thrombelastograph in two freshwater fish species. This also necessitated an investigation into the effects of different immobilization procedures on blood coagulation in freshwater fish when analyzing their haematological profiles. This technique therefore also has the ability to record the possible effects of added catecholamines and other stressor hormones on blood coagulation to determine the possible causes of stress resulting in accelerated clotting. Furthermore, several basic coagulation studies were also undertaken to identify both intrinsic and extrinsic coagulation factors and the aggregatory response of thrombocytes in fish blood. For this purpose, more than 1000 samples were collected and analyzed from the two freshwater fish species used in this study. The results are presented according to the relevant investigative stages followed.

4.1 Thrombelastography (TEG)

Before evaluating the results recorded, it is essential to explain the basic principles of operation of the **TEG** and the interpretation of the **TEG** as described by **De Nicola (1957)** and **Hartert (1971).**

4.1.1 Principles of operation - Fig. 2

The thrombelastograph is a mechanically operated device that provides a continuous visual kymographic observation of blood during all phases of coagulation .The resultant recording is termed a **thrombelastogram (TEG - Fig. 3).** The instrument consists of two cylindrical stainless steel cuvettes which are thermostatically maintained at **37°C.** The cuvettes are connected to a moving device that rotate them to and fro through an angle of **5°** around a vertical axis. A piston is lowered by means of a torsion wire into the cuvette, leaving a space of **1 mm** between the cuvette and the piston. The torsion wire in turn, is connected to a heating stylus that writes on a heat sensitive paper, moving at a speed of **2 mm** per minute. If unclotted blood is introduced into the cuvette, the torsion wire remains motionless, but as clot formation proceeds between the piston and the cuvette, the rotation of the torsion wire increases, thereby recording a positive and negative deflection of the stylus on the paper to confirm the results of the coagulation tests.

4.1.2 Evaluation of the thrombelastogram (De Nicola, 1957)

The **TEG** displays certain characteristics that are used to evaluate the clotting

process **(Fig. 3)**. These include:

4.1.2.1 The r time

The **r time** corresponds to the straight line extending from the starting point on the recording chart to the point where the amplitude reaches 1 mm. During this time the piston remains motionless in the fluid specimen. This distance, converted to time, corresponds with clotting time or the rate of thromboplastin generation. It therefore represents the participating factors in the intrinsic coagulation pathway. Thus, lengthening of the **r time** represents an inherited defect in thromboplastin generation, ***eg*** classical hemophilia or an acquired defect such as increased levels of circulating anticoagulants. Conversely, a shortening of the **r time** appears to be associated with accelerated clotting or hypercoagulability, shock, the first stage of disseminated intravascular clotting and uremia.

4.1.2.2 The k time

The **k time** corresponds to the time taken from the end of the **r time** to a point where the amplitude reaches a maximum of **20 mm.** This represents a measure of the rapidity of fibrin formation or the speed at which a clot of a certain solidity is attained. This time is responsive to the intrinsic plasma and thrombocyte clotting factors. Coagulation defects associated with prolonged **k times** include deficiency of intrinsic factors **(XII, XI, IX)** or hypocoagulable blood. Reduced **k times** are always associated with hypercoagulability.

4.1.2.3 The r + k values

The arithmetical summation of **r and k times** is equivalent to the clotting time. Since the **r time** represents the generation of thromboplastin and the **k time** the formation of thrombin, the total value of these two parameters therefore reflects the first and second phases of blood coagulation. It represents the so-called kinetic coagulability phase **(Raby & Vaivel, 1967).**

4.1.2.4 The maximum amplitude (Ma)

De Nicola (1957) and **Marchal *et al* (1961)** defined the maximal amplitude as being the maximal elasticity of the clot. This is influenced by the dynamic properties of fibrin, calcium, factor **XIII,** certain plasma factors and to a significant extent by platelet function. A decreased amplitude can be associated with thrombocytopenia, platelet defects, decreased fibrinogen levels, fibrinogen and fibrin breakdown products of fibrinolysis and increased retractability of the clot. An increased **Ma** is usually found in hypercoagulable conditions (**De Nicola, 1957; Marchall *et al* 1961).**

4.1.2.5 The index of thrombodynamic potential (ITP)

The **r and k values** represent the kinetic coagulability as discussed above. The dynamic coagulability is dependent on platelet function, fibrinogen levels, factor **XIII** and the haematocrit. This is expressed as the index of thrombodynamic potential and is calculated from the ratio **EMX\k. Van Vliet (1981)** suggested that the calculated **ITP** values of **TEG's** for similar or

interspecies differences may differ slightly between research laboratories due to variations in the application of the technique. It is therefore emphasized that each research laboratory should establish its own range of standard values for all the different parameters.

4.1.2.6 Thrombelastographic index (TI)

Serial determination of plasma fibrinolytic activity, anti-trypsin and anti-thrombin levels can be calculated from the **TEG** parameters according to the following formula: **(r + k)100 ÷ Ma**. The most important conclusions from a physio-pathologic point of view, are usually drawn from the fundamental values of **r , k and Ma** values.

4.1.3 Effects of anaesthesia on blood coagulation in freshwater fish.

Several anaesthetization procedures have been described by **Smit *et al* (1979)** with specific reference to freshwater fish. Apart from the evaluation of the potency of **MS 222 (Sandoz)** and **neutralized MS 222**, the effects of these anaesthetics on the haemotological profiles of freshwater fish were also analyzed. In this study, further attempts were made to obtain additional information on the effects of anaesthesia on blood coagulation prior to assessing the effects of stress on the clotting process.

4.1.3.1 Effects of neutralized MS 222 on blood coagulation

Fig.4 shows the **TEG** patterns recorded for unstressed fish anaesthetized with

neutralized **MS 222** for ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** respectively. A typical wine glass pattern is obtained which corresponds to that of human blood as indicated in **Figure IVb.** According to **Nel (1976), ITP** values for normal human **TEG's,** should fall within the range of **19 - 23.** Such values were also recorded for the two species studied. These results therefore confirm the effective use of **neutralized MS 222** for both freshwater fish species used in this investigation **(Table 1).**

Significant interspecies differences **($p < 0.005$)** for both ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** were evident. These resulted largely from the statistical differences in the **mean r , k and Ma values.**

4.1.3.2 Effects of electronarcosis on blood coagulation.

The patterns indicated in **Fig.5** explain the **TEG's** recorded when fish were electronarcotized. Such recordings were similar to those obtained with **neutralized MS 222.** In general, however, lower mean values for all the **TEG** parameters measured were obtained, excluding **ITP values,** when compared with those for **neutralized MS 222. ITP** values were significantly higher. In addition, the **TEG** values measured for ***Schilbe (S) mystus depressirostris,*** were significantly reduced when compared with those for ***Clarias gariepinus.*** These observations suggest that blood coagulation in the two freshwater fish species investigated, involves the same basic processes as those of human blood. **Table 2** lists the **TEG** values for both species.

4.1.3.3 Effects of natural MS 222 on blood coagulation.

The **TEG** recordings illustrated in **Fig. 6,** indicate the results obtained when fish were anaesthetized with **MS 222** in its natural acidic form. Comparison of these results with those in **Figs.4 and 5,** indicated marked differences. The **TEG,** in this case, was characterized by **long r, k and decreased Ma and ITP values (Table 3).** Significant interspecies differences **($p < 0.005$)** were recorded for the two species. As indicated in **Table 3,** butter catfish recorded significantly higher mean values when compared with those for the sharptooth catfish. These results suggested a possible decrease in clotting ability. Visual observation of the blood suggested that these different recordings compared to those obtained for neutralized MS 222, resulted from partial clotting prior to the addition of the blood to the cuvettes.

4.1.3.4 Effects of MS 222 added to fish blood.

Confirmation of the above-mentioned observations was achieved by recording the effects of **natural MS 222,** in relatively high concentrations, added directly to blood sampled from unstressed fish. This resulted in the recording of a similar, but more pronounced hypocoagulable **TEG** recording **(Fig. 7). Ma** values **($p < 0.005$)** were significantly reduced in both species, with butter catfish having slightly higher values **(Table 4)** than ***Clarias gariepinus,*** when compared to standard values. This suggested that natural MS 222 produced a false hypocoagulable state that may explain the different haematological effects recorded by **Smit *et al* (1979)** for natural MS 222 anaesthetized fish.

4.1.3.5 Effects of neutralized MS 222 on blood coagulation without the use of anticoagulant.

These results are presented in **Fig. 8** for ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** respectively. The **TEG** recording was characterized by shorter **r and k values** associated with an **increase** in **maximal amplitude** for both species. Butter catfish exhibited higher values than sharptooth catfish **(Table 5)**. Mean values for various clotting parameters in both species also showed significant differences **($p \leq 0.005$)**, except for the dynamic coagulability phase **($p \leq 0.05$)**. These results were therefore associated with hypercoagulability and also confirmed by the reduced **r + k values**. The latter reflected the total coagulation time **(kinetic coagulability)** of the blood in both fish species studied.

4.1.3.6 Effects of natural MS 222 on blood coagulation without the use of an anticoagulant.

The results for both ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** are presented in **Fig. 9** and **Table 6**. The **TEG** patterns indicated **shorter r and k values** with an **increase** in **maximal amplitude**. No significant interspecies differences were recorded for the mean r values. These observations therefore suggested accelerated clotting as outlined in **section 4.1.3.5 .**

4.1.4 The effects of stress on blood coagulation in freshwater fish.

The effects of stress on blood clotting in both fish species were determined

by thrombelastography. The **TEG,s** were recorded for different simulated stress situations as indicated by **Figs. 10, 11** and **12.**

4.1.4.1 The TEG recordings of stressed fish without the use of an anticoagulant.

Fig. 10 & Table 7 indicate the results obtained when fish were stressed and blood sampled without **TSC.** These particular **TEG's** were characterized by **shorter r and k values** associated with an **increase** in **maximal amplitude.** The **dynamic coagulability** of the blood was also **significantly increased** when compared with those recorded for unstressed fish. Significant interspecies differences **($p < 0.05$)** were thus recorded. Another important observation was the very low values recorded for the total coagulation times or kinetic coagulability phase of the blood in both species. These observations corresponded with hypercoagulability recordings observed in human blood. These results also did not suggest significant differences when compared to those obtained when natural **MS 222** was used without the use of the anticoagulant **TSC.**

4.1.4.2 The TEG recordings for acutely stressed freshwater fish.

Fig. 11 presents thrombelastogram patterns for acutely stressed ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris.*** In both instances, significantly **reduced r and k values ($p \leq 0.001$)** were obtained in all cases when compared with those obtained for unstressed fish. These observations

suggested hypercoagulability of fish blood. No significant interspecies differences in **TEG** patterns and values were obtained for both species. Mean values for various parameters were slightly higher in ***Schilbe*** as compared to those in ***Clarias*** (**Table 8).** The kinetic coagulability phase **(r + k)** also suggested reduced values when compared with those obtained for unstressed fish. These observations confirm possible stress effects on fish blood coagulation.

4.1.4.3 The TEG recordings for chronically stressed fish.

Chronically stressed fish produced similar effects to those of acutely stressed fish **(Fig. 12).** The mean values, as indicated in **Table 9,** were slightly lower as compared to those obtained for acutely stressed fish, thus suggesting that the rate of clotting was higher in chronically stressed fish than in acutely stressed ones. Both acute and chronic stressed fish produced hypercoagulable blood.

4.1.5 Effects of added single or combined stressor hormones on blood coagulation.

In order to determine the cause of hypercoagulability during stress situations, it was decided to evaluate the effects of different stressor hormones individually and in combination when added to the blood of unstressed fish. It was hoped that in this way the cause of hyper and hypo coagulable blood could be determined.

4.1.5.1 Effects of cortisol on blood coagulation

When cortisol was added to the blood of unstressed fish, the **TEG** was characterized by **long r and k** values with **decreased maximal amplitude** in both ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** as indicated by **Fig. 13** and **Table 10.** These observations corresponded with results obtained for natural MS 222 anaesthetized fish. Careful observation of such samples indicated that these recordings may result from accelerated or premature clotting **similar to the addition of MS 222 to the blood.** In addition, significant interspecies differences **($p \leq 0.001$)** were recorded for the **r, k and ITP values** in the two species studied.

4.1.5.2 Effects of adrenaline on blood coagulation.

Fig. 14 and **Table 11** show the results obtained when adrenaline was added to the blood of unstressed fish. **Short r and k values** were recorded as well as **decreased Ma values.** The **ITP** values were also **lower** than those obtained for unstressed fish. These results conform to those obtained for cortisol, which indicated the partial clotting of blood. Significant interspecies differences **($p \leq 0.001$)** were also observed for the two species.

4.1.5.3 Effects of noradrenaline on blood coagulation.

Noradrenaline alone did not produce a significant change in the **TEG** recordings for both species when it was added to the blood of unstressed fish. It corresponded to **TEG** recordings for unstressed fish. Such **TEG**

patterns are represented in **Fig. 15,** whereas the mean values for the different parameters measured, are listed in **Table 12.** Significant intraspecies differences were recorded for the different parameters measured in the two species when compared with the results recorded for neutralized MS 222. Meaningful interspecies differences were also common for all **TEG** parameters in the two species studied.

From the observations made above on the effects of individual stressor hormones to the blood, it was decided to evaluate the effects of adding different combinations of stressor hormones to the blood of unstressed fish. As indicated previously, three different effects were recorded for the three hormones. Such recordings for a combination of hormones were essential to describe their possible combined role during stress.

4.1.5.4 The combined effects of adrenaline and cortisol on blood coagulation in freshwater fish.

These results are illustrated in **Fig. 16** and **Table 13** for ***Clarias gariepinus*** and ***Schilbe(S)mystus depressirostris.*** The **TEG's** for the two species were generally characterized by **short r and k times** and **high Ma values** that corresponded to those for hypercoagulable blood. The total coagulation times **(r + k)** were reduced significantly in both species, with butter catfish showing significantly higher values than sharptooth catfish. These results were supportive of hypercoagulable blood, similar to those obtained for stressed fish.

4.1.5.5 The combined effects of cortisol and noradrenaline on blood coagulation.

Fig. 17 and **Table 14** show the **TEG** patterns recorded when cortisol and coradrenaline are added in combination to the blood of unstressed fish. The patterns produced in this case were similar to those observed when natural MS 222 was added to the blood. This observation therefore also suggest accelerated or premature clotting or hypercoagulable blood with significant interspecies differences. The recorded results therefore suggested that, under severe or acute stress conditions, these hormones may be released as primary factors which, in turn, may produce minute clots with resultant death.

4.1.5.6 The effects of adrenaline and noradrenaline on blood coagulation.

Fig. 18 and **Table 15** show the **TEG's** and parameters recorded when adrenaline and noradrenaline were added to the blood of unstressed fish. The results obtained were virtually similar to those for cortisol and noradrenaline, but with significant differences. Visual observation of the samples indicated the presence of tiny clots, possibly resulting from **DIC**.

4.1.5.7 Combined effects of cortisol, adrenaline and noradrenaline on blood coagulation.

Smit (1990) reported that the level of all stressor hormones increased during any stress situation. Since no direct comparison with stressed TEG's could be

obtained for the individual or combined hormones added to the blood of unstressed fish, it was decided to record such effects when added to the blood at different concentration levels. When cortisol, adrenaline and noradrenaline were added in equal quantities to the blood of unstressed fish, similar recordings to those for cortisol and noradrenaline were obtained **(Fig. 19)**. This corresponded to premature or accelerated clotting or disseminated intravascular clotting.

4.1.5.8 Combined effects of cortisol, adrenaline and a low concentration of noradrenaline on blood coagulation.

When low levels of noradrenaline were combined with relatively high levels of cortisol and adrenaline and added to the blood of unstressed fish, a **typical TEG pattern identical to that recorded for stressed fish,** were obtained **(Fig. 20 & Table 17).** This suggested that all three stressor hormones were involved in a similar combination during stress.

4.2 The effects of stress on Lee White Clotting times of fish blood.

The results obtained for whole blood clotting times in ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** when exposed to chronic and acute stress, are listed in **Table 25.** Mean atraumatic whole blood times for unstressed fish were 141.29 ± 0.82 seconds and 128.57 ± 0.56 seconds in Sharptooth catfish and butter catfish, respectively. However, a significant decrease **($p \leq 0.001$)** to 62.78 ± 1.60 and 53.52 ± 1.31 seconds was observed respectively when both ***Clarias*** and ***Schilbe*** were acutely stressed. Significant

intraspecies differences **($p \leq 0.001$)** were also recorded between unstressed blood samples and chronically / acutely stressed samples in both species. ***Clarias gariepinus*** showed higher mean values in all cases as compared to ***Schilbe (S) mystus depressirostris.***

4.3 The effects of stress on prothrombin times in freshwater fish.

Prothrombin times for fish blood were recorded at room temperature with thromboplastins prepared from ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** brains as well as **Ortho rabbit brain thromboplastin**. These results are listed in **Tables 26 - 28** respectively.

The addition of ***Clarias gariepinus*** brain thromboplastin to the plasma of both fish species, produced higher prothrombin times when compared with ***Schilbe*** thromboplastin. Plasma obtained from acutely and chronically stressed fish produced significant decreases **($p \leq 0.001$)** in prothrombin times when brain thromboplastin prepared from both species were used. The addition of plasma from sharptooth catfish and butter catfish to **ORTHO rabbit brain thromboplastin,** produced significantly higher prothrombin times of 128.84 ± 2.21 and 98.41 ± 0.11 in unstressed fish **(Table 28).** In stressed fish, a significant decrease in prothrombin times were obtained. These times, however, were higher when compared to those recorded for fish brain thromboplastins.

4.4 Intrinsic and extrinsic blood coagulation factors

Clotting times for the different intrinsic and extrinsic factors for the two species, are listed in **Table 29**. In general, all clotting times for these factors were lower in ***Schilbe*** than in ***Clarias.*** Also significant in the case of ***Clarias gariepinus,*** was that the extrinsic clotting factors produced significantly shorter clotting times when compared with the intrinsic factors. Furthermore, the differences in clotting times for both the intrinsic and extrinsic factors in ***Schilbe,*** were not so significant as the case with ***Clarias.*** Also, it appeared as if the clotting times for the two systems were reversed in ***Schilbe.*** In both species, **Factor X** occupied a pivotal position in terms of significantly shorter clotting times. These observations were supportive of a well developed clotting system in the two fish species studied.

4.5 Thrombocyte aggregation functions in the blood of *Clarias gariepinus* and *Schilbe (S) mystus depressirostris.*

The aggregation responses recorded by the addition of adenosine diphosphate **(ADP)** to the blood of ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** are shown in **Figs. 21** and **22** respectively. Both figures suggest that **ADP produced irreversible aggregation** when **15 µl** was added to the blood. The addition of **2.5 µl of ADP** did not produce any significant aggregation responses. When the fatty acid, sodium arachidonic acid **(AHA),** was added to the blood of these two species, similar but more severe and significant responses were recorded when compared with **ADP.** In both species, complete aggregation was observed when higher concentrations of

ADP and **AHA** were added to the blood of ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris.*** The addition of **2.5 µl AHA** produced reversible aggregation in both species as indicated in **Figs. 23 and 24.** However, aggregation was not induced by the addition of **Decosahexaenoate** to the blood of the two fish species **(Figs. 25 & 26)**. When low concentration of **epinephrine (E)** was added to the blood of both species no aggregatory response was observed. However, an increase in the concentration of epinephrine resulted in aggregation of the thrombocytes for both species **(Figs. 27 & 28)**. These observations therefore suggest that some similarities were possible with the platelet functions in higher vertebrates.

CHAPTER V

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CHAPTER 5 *DISCUSSION*

Introduction

Investigations into the blood physiology of freshwater fish have been gaining international recognition for many years, mainly due to the diagnostic value of haematological profiles for determining their health status. Such clinical observations can also be used to identify the sublethal effects of stress. The information collected in this way can therefore provide valuable contributions toward a greater understanding of physiological responses of fish to a changing external environment. This results from the close association of the circulatory system with the external environment. One stress response that received relatively little research attention, was the effects of stress on the very sensitive blood clotting system. In mammals, this system appeared to be a dynamic process undergoing alterations and adjustments to changing environmental conditions **(Schmer & Strandjord, 1973)**.

The clotting process basically involves the interaction of protein factors that result from tissue injury or trauma **(Guyton, 1986)**. In this regard, several reports are available on the successful use of routine screening tests for human blood as an alternative for the evaluation of blood coagulation in fish **(Van Vliet *et al* 1985; Kawatsu, 1986; Smit & Schoonbee, 1988)**. In spite of the progress made in this particular field, various problems related to clotting of blood in fish are still experienced in the laboratory. The most important of these involves the presence of tiny blood clots when sampling blood with sufficient anticoagulant for haematological assays in certain species. **Smit (1980)** suggested that this resulted from the inadequate use of

anticoagulants. **Van Vliet *et al* (1985),** on the other hand, indicated that this occurred as a result of poor blood sampling techniques. Our laboratory experiences indicated that blood samples collected from ***Clarias gariepinus*** were found to clot, in spite of serious attempts to collect atraumatic samples with sufficient anticoagulant. Visual observations of samples from several other indigenous species exposed to stress, indicated the presence of tiny clots, resembling those for disseminated intravascular coagulation. This evidence suggests that a careful evaluation of the clotting system in fish may resolve the problems identified. Furthermore, these findings also did not consider the general use of commercially available polytop vials for blood sampling and analysis in fish. The suitability of these to standardize blood clotting tests in field and laboratory experiments is therefore essential. The major aim of this study, however, remained the evaluation of stress effects on blood coagulation as a means of explaining mortalities arising from certain stress situations. This is specifically related to the mortalities resulting from catching and transport of live fish to the laboratory. Physical disturbance and handling of fish are common, often unavoidable, features of many experimental investigations. Also, intensive culture of fish requires the use of various handling and transport procedures which ultimately cause stressful reactions. These, in turn, are also responsible for a high mortality rate in certain indigenous fish species. The primary cause of such mortalities was ascribed to osmoregulatory disturbances **(Hattingh, 1976)** These observations, however, did not consider stressful reactions of other functional systems. Consequently, the responses of fish to this form of stress have attracted considerable attention.

It is also well-known that handling and anaesthesia are known to induce changes in the physiological status of a fish **(Wedemeyer, 1970; Sovio & Oikari, 1975). Smit et al (1979)** reported that anaesthesia with **MS 222 (Sandoz)** resulted in chemical stress as evidenced by changes in the haematological profiles of such animals when compared with those recorded for **neutralized MS 222.** Furthermore, no evidence was submitted to suggest possible effects of chemical anaesthesia on blood coagulation. Observations made in this laboratory, therefore, suggested a re-evaluation of procedures for immobilization of fish in an attempt to avoid the consequences of stress on blood coagulation. In this particular investigation, only certain biochemical factors involved in blood coagulation were used to study some effects of stress on the normal clotting processes.

Fish thrombocytes also differ histologically from platelets of mammalian blood **(Pieterse, 1982).** Their functions, however, appear to be similar. The latter author indicated that fish thrombocytes are true cells with different shapes and sizes. The reasons for such differences are not well-known. This naturally necessitated the evaluation of the aggregatory behavior of fish thrombocytes to identify their role during coagulation under standard conditions as well as during stress. It was therefore evident that the preparative steps taken in the laboratory for the analysis of fish blood still posed serious problems.

The primary aim of this investigation was therefore to study the effects of stress on blood coagulation in order to reduce the high mortalities of fish during routine laboratory procedures. These could serve as a basis for improving and standardizing laboratory

experimental procedures whereby a more realistic interpretation of the actual physiological status of fish may be obtained.

5.1 Blood coagulation

Haemostasis in humans and higher vertebrates are well documented for medical diagnosis under various conditions of abnormality, disease and surgical intervention. In this regard, information on blood coagulation in fish is limited, although **Doolittle & Surgenor (1962)** and **Lewis (1972)** indicated that the blood coagulation process in fish resembled that of humans and other higher vertebrates. Until recently, the possible occurrence of accelerated or retarded clotting under stress conditions was never considered. **Van Vliet (1981)** used the thrombelastograph to indicate interspecies clotting differences in fish. They also investigated species specific clotting activities during stress, but did not offer any acceptable suggestions to explain such differences. In this investigation, an attempt was therefore made to compare whole blood clotting times of fish with those for higher vertebrates in order to identify possible differences. In addition, an evaluation of blood coagulation using more modern techniques, such as aggregometry, were also used as a basis to confirm whether this process was indeed similar to that occurring in higher vertebrates and to identify the differences that may be responsible for the changes that were recorded during this investigation. Furthermore, it was essential to record the possible existence of extrinsic and intrinsic blood clotting factors with the use of routine screening tests applied for human blood. Such results would indicate clearly whether the clotting fundamentals in fish were similar to those of

higher vertebrates. It was also essential to determine the different roles of these factors before and after stress. The results presented in this study will be discussed in the order of the various analytical steps followed to eventually explain the results recorded.

5.1.1 Whole blood clotting times

A number of routine tests are usually applied in human blood coagulation investigations to screen possible bleeding disorders. The value of most of these tests have been shown to be of great medical significance as previously explained. Only limited investigations of this nature have thus far been undertaken for freshwater fish. Also very little evidence in this regard is available for indigenous South African fresh water fish species. **Lee and White whole blood clotting times** were determined for ***Oreochromis mossambicus*** and ***Cyprinus carpio*** by **Van Vliet *et al* (1985).**

Cyprinus carpio is an exotic species introduced into South African water bodies. These species, apart from an angler's view point, is of no real economic significance to South Africa. Such tests have to be undertaken on indigenous species to support a local and economically viable freshwater fish industry. The procedures for testing the **bleeding method of Ivy (Dacie & Lewis, 1984)** have also not been used to identify intraspecies and interspecies differences for indigenous fish species.

From the above observations, it can be concluded that the estimation of whole blood coagulation strategies has been of limited value in general fish

culture. It may, however, be extremely useful to determine and explain the occurrence of unexpected mortalities in freshwater fish. Great care should therefore be taken when using such techniques, since small amounts of thrombin, resulting from the traumatic sampling of blood, may affect the results obtained negatively **(Van Vliet *et al* 1985)**. Such techniques are therefore extremely sensitive, since poor blood collecting procedures may cause unexpected fibrinogen clotting effects that may contribute to false results **(Dacie & Lewis, 1984)**. Such observations were indeed confirmed by **Van Vliet (1981)**.

The whole blood coagulation time is a non-specific test, because deficiencies in almost any factor can lead to prolonged coagulation times. In practice, however, prolonged times are usually associated with factors **VIII, IX or XII** deficiencies **(Dacie & Lewis 1984)**. In this investigation the **Lee and White blood coagulation test** was included to determine the time taken by fish blood to coagulate under stress and non-stressful situations and also whether stress may have lethal consequences. Extreme care was therefore taken to collect blood atraumatically and to standardize the technique as accurately as possible. It was also observed that blood sampling by cardiac puncture often resulted in the unexpected death of fish, possibly as a result of severing the cardiac conducting system of the heart during sampling. Venipuncture was found to be a better sampling method in terms of fish survival rates after blood sampling. Furthermore, blood sampling by venipuncture prevented the occurrence of unwanted blood clotting when collecting samples in the field as opposed to sampling by cardiac puncture. Secondly, this technique also proved to be more effective in demonstrating the results of acute and chronic

stress on whole blood clotting times. Such results can therefore be used to explain fish mortalities resulting from different stressful handling procedures in laboratory aquaria.

Clarias gariepinus presented significantly longer clotting times for unstressed fish than ***Schilbe (S) mystus depressirostris.*** The effects of chronic and acute stress on blood clotting, showed a remarkable decrease in coagulation times. **Cassillas & Smith, (1977)** also reported reduced clotting times of fish blood under stress conditions. **Van Vliet (1981)** confirmed such results with the use of thrombelastography. The **TEG's** obtained differed from the standard wine-glass shaped recordings when fish were exposed to different stress situations. **Smit & Schoonbee (1988)** also suggested that stress may result in increased disseminated intravascular coagulation, which can be fatal in some fish species. Visual observations on fish subjected to different types of simulated stress situations under controlled laboratory conditions, indicated the appearance of white, necrotic patches on the skin of certain fish species after a few days. These may be caused by the presence of tiny blood clots or disseminated intravascular coagulation that block minute blood vessels to the tissues. The occurrence of the latter may possibly inhibit blood flow to the tissues or vital organs sufficiently to cause death. From these observations, it can be concluded that the **Lee and White blood clotting times** may be a useful indicator for establishing standard and abnormal coagulation times for fish blood. The effects of external stressful and other stimuli may therefore also be used to identify specific responses on blood clotting activities.

The results obtained in this investigation, clearly suggested that the whole blood clotting times of the species studied, were significantly longer than those known for human blood. These were also confirmed by the results of **Van Vliet (1981)** when evaluating the clotting times for ***Cyprinus carpio*** and ***Oreochromis mossambicus.*** Several factors may be responsible for such differences. These include, amongst others, the differences in circulating levels of the clotting factors as well as the differences associated with the nature of platelets in higher vertebrates and the fact that fish have true cellular thrombocytes. These will be discussed again when considering other studies on the coagulation system of the two species used during this investigation.

The most significant observations made in this study on whole blood clotting times, was a significant reduction of approximately 60% in whole blood coagulation times when fish were subjected to chronic stress. Furthermore, acute stress reduced these times further to 17% in ***Clarias gariepinus*** and 8.5% in ***Schilbe (S) mystus depressirostris*** when compared with their respective standard results of unstressed fish. These observations indicated that, acute stress produced a more significant effect than chronic stress and that such differences were more noticeable in ***Schilbe (S) mystus depressirostris***. This suggested that, the latter species was more sensitive to stress as judged by the whole blood clotting times recorded. It also appeared that the triggering mechanism responsible for such actions in the butter catfish, are more sensitive than those in ***Clarias gariepinus.*** These may be related to higher circulating clotting factors, the levels of circulating stressor hormones following stress, thrombocyte numbers following stress as well as

the levels of **ADP** and other aggregating agents responsible for thrombocyte aggregation in the circulating blood following stress. Thus, stress evoked a rapid mobilization of the haemostatic mechanism to prepare for a fluid loss rather than a blood loss. The latter is derived from the results of **Hattingh (1976)** following mortalities resulting from transport stress. **Smit (1990)** indicated that catecholamine levels rose during stress and that the above-mentioned blood changes might be possible following stress. This author also suggested that such changes were more significant in ***Oreochromis mossambicus***. In the light of these suggestions, such possibilities should be considered along with the findings recorded in the following sections to be discussed.

5.2 Thrombelastography of fish blood.

TEG provides valuable information regarding the presence and activity of both the intrinsic and extrinsic factors involved in blood coagulation in higher vertebrates. However, the major importance of this technique lies in the evaluation of the intrinsic coagulation system, since intrinsic thromboplastin generation is more complex than that of the extrinsic system **(Biggs, 1976)**. **Van Vliet *et al* (1985** a,b) indicated that the process of blood coagulation in fish was similar to that of humans and mammalians, involving the same three basic processes, **ie** extrinsic and intrinsic thromboplastin generation, the conversion of prothrombin to thrombin and the formation of fibrin from fibrinogen. This technique of thrombelastography is ideally suitable for confirmation of the different clotting stages in fish blood. Based on these observations, the latter author suggested that thrombelastography can also

be effectively used as a diagnostic tool for assessing stress in fish.

5.2.1 Effects of anaesthesia on blood coagulation.

Until recently, the use of chemical anesthetics, such as **MS 222 (Sandoz)**, and **benzocaine hydrochloride** were widely recommended for immobilizing fish **(Ferreira *et al* 1979; Smit, 1980)**. Although these substances anaesthetized the animals effectively and thereby minimized handling stress and other stressful conditions, they also produced side effects on the blood physiology of fish **(McFarland, 1959; Smit, 1980)**. Also, handling and anaesthesia are known to induce changes in the physiological status of fish **(Wedemeyer, 1970; Sovio & Oikari, 1975)**. It was also reported by **Smit *et al* (1979)** that anaesthesia with **MS 222 (Sandoz)** resulted in chemical stress as was evidenced from the haematological profiles of the animals used. However, all the evidence provided in the investigation of the latter authors, did not consider the possibility of anaesthetic effects on blood coagulation. The many unknown factors influencing the **TEG**, as well as those unknown aspects of fish blood coagulation, resulted in the emphasis being placed on recording a **TEG** pattern that corresponded with typical wine-glass shaped recordings usually obtained with normal human blood **(Hartert, 1971)**. When neutralized **MS 222 (Sandoz)** was used, a typical **TEG** wine glass pattern was recorded for the two different fish species used during this investigation. These results also corresponded to those for human blood **(Schneider, 1962)** and the **TEG's** recorded by **Van Vliet *et al* (1985)** for ***Oreochromis mossambicus*** and ***Cyprinus carpio.*** According to **Nel (1976), ITP** values for normal human **TEG's**, should fall within the range of 19-23. The ITP values

recorded with the use of neutralized MS 222 for ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** in this investigation showed no significant interspecies differences, nor any significant differences with those recorded for human blood. This confirmed the effective use of **neutralized MS 222** for immobilizing fish.

On the basis of these results, it was evident that blood coagulation in the two freshwater fish species studied, involved the same basic processes as those for human blood. Furthermore, distinct differences occurred between whole blood clotting times and **TEG** clotting times for fish blood and these may be interpreted as incorrect. These differences are ascribed to different testing procedures. Whole blood clotting times involved the development of a natural clotting process, whereas the clotting times recorded with the **TEG** was based on the recalcification of the blood. It is concluded that the clotting process in the two freshwater fish species studied, also involved thromboplastin generation, a prothrombin complex and the conversion of soluble fibrinogen to fibrin whereby a clot is formed. The above information provided sufficient evidence for the presence of a well developed coagulation system in the blood of ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris.***

When natural, acidic **MS 222 (Smit, 1980)** was used to anaesthetize fish, the **TEG** pattern changed completely and conformed to those obtained for a decreased clotting ability **(Fig. 6 and Table 3)**. The **TEG's** in this case was characterized by **long r and k times** and **decreased Ma values. Marchal *et al* (1961)** reported that a decreased amplitude can also be associated with

thrombocytopenia, platelet defects, decreased fibrinogen levels and increased retractability of the clot. However, visual observations of the blood used in this study, indicated that this resulted from partial clotting of the blood prior to filling the **TEG** cuvettes. Such changes were also noted in the samples that were not used for **TEG** recordings. To confirm such observations, it was decided to record the effects of **natural MS 222** added to blood sampled from unstressed fish. This resulted in a similar, but more pronounced effect **(Fig. 7).** The **r** and **Ma values** in both species were significantly reduced, with butter catfish recording more significant and more effective differences related to stress sensitivity when compared with similar values for those of the sharptooth catfish **(Table 4; Fig. 7). De Nicola (1975)** and **Hartert (1971)** indicated that **shortening** of the **r time** appeared to be associated with accelerated coagulability or hypercoagulability, shock and the first stage of disseminated intravascular clotting. This suggested that natural MS 222 produced a false hypocoagulable state that may explain the haematological changes recorded by **Smit *et al* (1979)** for **natural MS 222** anaesthetized fish. It can be safely concluded that, chemical stress was the causative agent, since it was shown that **natural MS 222** had a direct effect on the clotting process in the two freshwater fish studied. The possible suggestion to explain these results, may be associated with the acidity of natural **MS 222,** since similar visual observations were made when adding a low volume and concentration of acetic acid to the blood of unstressed fish. These observations suggests that **natural MS 222** should not be used as an anaesthetic for freshwater fish when undertaking blood physiological studies and that further investigations are essential to confirm the observations made.

5.2.2 Effects of anaesthesia without the use of anticoagulant

Van Vliet *et al* (1985) recorded **TEG** patterns that corresponded with hypercoagulable blood when using relatively low levels of tri-sodium citrate **(TSC)**. In this investigation an attempt was also made to look into the effects of **neutralized** and **natural MS 222** anaesthesia without the use of **TSC** as an anticoagulant. With **neutralized MS 222, r and k times** were significantly reduced **($p \leq 0{,}001$)**, whereas **Ma** values increased significantly, thus indicating hypercoagulable blood **(Table 5; Fig. 8)**. Similar, but more pronounced results were recorded with the use of **natural MS 222 (Figure 6; Table 9)**. These observations confirmed the results of **Van Vliet *et al* (1985)**, suggesting that accelerated clotting occurred as a result of higher calcium levels in the blood following recalcification. This implied that coagulation continued normally under these conditions. The interspecies differences recorded may therefore result from higher calcium levels in the blood of butter catfish **(Smit, 1990)**. Thus, accelerated clotting or hyper coagulated blood were more significant in ***Schilbe (S) mystus depressirostris,*** thereby explaining the differences between the **Lee - White Clotting times** and the recalcification clotting times for the **TEG** recordings of the two species. Recalcification promoted and accelerated polymerization of fibrin to produce a greater maximal deflection similar to a stressed **TEG**. Upon recalcification of the blood, the coagulation process was accelerated due to the presence of excess calcium. **Doolittle & Surgenor (1962)** and **Lewis (1972)** reported that increased concentrations of calcium resulted in a marked decrease in the coagulation time of fish blood. Their results corresponded with the

observations made in this study. It is therefore important to realize, that the use of thrombelastography as a diagnostic tool, requires accurate and precision of operation to record meaningful results.

5.2.3 Effects of electronarcosis on blood coagulation.

Barham (1987) described the use of electronarcosis as a suitable alternative to immobilize fish for the evaluation of haematological profiles under laboratory conditions. However, the role of electronarcosis on blood coagulation was never determined to qualify this statement. Furthermore, this technique appeared to be cheaper and also easier to use when compared with chemical anaesthesia. The few studies to date also suggested that recovery was uneventful and possibly less stressful when compared with that of chemical anaesthesia **(Madden & Houston, 1976; Barham *et al* 1987).** When the fish were subjected to this type of anaesthesia, the TEG pattern recorded **(Fig. 5; Table 2),** resembled the wine-glass shape similar to that obtained for neutralized **MS 222.** These observations confirm that, electronarcosis is an equally suitable and effective method for immobilizing fish when compared to the use of neutralized MS 222. However, the reasons for such similar recordings can not yet be explained, since the one system is electrically operated, whereas, the other is chemically based. Such techniques therefore require further investigations. Furthermore, it is essential to note that **TEG** clotting times were not similar to those of Lee -White clotting times, since **TEG** times are based on recalcification of the blood, which is not the case with the **Lee - White clotting times.**

5.2.4 The effects of stress on blood coagulation

Stress in fish results mainly from an environmental stimulus which reduces the ability or capacity of a fish to maintain homeostasis, to survive, grow, and reproduce successfully **(Schreck, 1981).** The response of fish to stress has been considered to be similar in nature to the **General Adaptation Syndrome (GAS)** proposed for clinical diagnostic purposes **(Seyle, 1976).** It should be noted, however, that the **GAS** was initially proposed by **Seyle (1936)** as a working model. **Schreck (1981)** suggested that this concept might also be useful for the evaluation of stress in fish. The latter author also suggested that the physiological responses of fish to stress were determined by the nature of the stress itself. That is, specific stresses elicit specific responses. Physiological consequences of stress, include fright and physical disturbances, such as encountered during handling or crowding of fish. In this investigation, an attempt was made to study the possible effects of stress on blood coagulation in order to explain the possible occurrence of mortalities resulting from the formation of lethal blood clots associated with disseminated intravascular clotting under different stressful conditions. The **TEG's** recorded for both ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** were characterized by significantly shorter r **and k times** ($p \leq 0.001$) associated with **increased Ma values.** These observations corresponded to hypercoagulable blood. ***Schilbe (S) mystus depressirostris*** recorded slightly lower values as compared to those of ***Clarias gariepinus*** **(Table 7; Fig. 10).** When both fish species were stressed acutely and chronically, similar but more pronounced effects were recorded, thereby also confirming accelerated clotting or hypercoagulable blood **(Figs. 11 & 12;**

Tables 8 & 9). Smit *et al* (1990) reported on differences in catecholamine and cortisol levels for these species subjected to different stress conditions. In order to evaluate the possible endocrine stimulus for accelerated clotting or hypercoagulable blood during stress conditions, it was necessary to evaluate the effects of single and different combinations of stressor hormones added to the blood of unstressed fish, using neutralized MS 222 as immobilizing agent.

5.2.4.1 Effects of cortisol

When cortisol was added to the blood of unstressed fish, the TEG was characterized by **increased r and k times** associated with **decreased Ma values (Fig. 13; Table 10)**. The decreased Ma values can be attributed to a possible counteraction to fibrinolytic activity, thereby causing the clot to dissolve while still in the process of formation. Thus, apart from possible deactivation of the fibrinolytic system, it was also possible that reduced fibrinogen levels, resulting from disseminated intravascular clotting caused by cortisol, may also have contributed to the lesser **Ma** values. Clot solidity and elasticity would be reduced during stress conditions. Such effects were more pronounced in ***Schilbe (S) mystus depressirostris*** than in ***Clarias gariepinus.*** These observations corresponded with results obtained for **natural MS 222 anaesthetized fish.** Close observation of the blood in the cuvettes indicated that cortisol possibly contributed to accelerated clotting. It appeared to have a direct effect on the clotting system, perhaps involving energy utilization associated with the production of **ADP. Hattingh (1976)** clearly indicated the diagnostic significance of elevated blood glucose levels during stress in fish.

This suggested a concomitant increase in energy mobilization with resultant higher circulating **ADP** levels. However, during stress, other stressor hormones are also being released **(Smit *et al* 1990)**. It was also important to determine their individual effects on blood coagulation in samples from unstressed fish.

5.2.4.2 Effects of adrenaline

When adrenaline was added to the blood of unstressed ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris,*** significantly **decreased r and k times,** as well as **Ma values,** were recorded for both species **(Fig. 14; Table 11).** According to **Nicola (1957),** and **Marchall *et al* (1961),** the kinetic coagulability **(r + k)** of less than 20 and the index of thrombodynamic potential of more than 15, represented the first stage of disseminated intravascular clotting. Thus, these results also conformed to those obtained for cortisol, which suggested the partial clotting of blood possibly resembled that for disseminated intravascular clotting. Thus, accelerated clotting was observed, resulting possibly from the action of this drug on thrombocytes. However, complete aggregation or clotting was not achieved, thereby suggesting that some form of inhibition to this response also occurred.

5.2.4.3 Effects of noradrenaline

Noradrenaline alone did not show a great change in the **TEG** recording when added to the blood of the two fish species studied. It did, however, correspond to that recorded for blood in unstressed fish or fish anaesthetized

with **neutralized MS 222.** As a single hormone added to the blood, it had no significant effects on the blood clotting process, perhaps due to the fact, that, this hormone had no notable effects on thrombocyte aggregation. Also, the fact that, several stressor hormones are being released during stress conditions, hint the possibility that the combined effects of two or more stressor hormones may produce different effects. It was important to study, also, such possible effects in order to determine their possible role during stress. Comparison of such results with those obtained under stress conditions, should be able to confirm their contribution to accelerated clotting.

5.2.4.4 Effects of cortisol and adrenaline

When cortisol and adrenaline were added to the blood of unstressed ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris,*** the **TEG** patterns were characterized by **short r and k times** and **increased Ma values (Fig. 16; Table 13),** similar to those recorded for stressed fish. These results corresponded with those for hypercoagulable blood, thus further confirming their contributory role during stress in fish. It does, however, not explain their possible effects on the role of thrombocytes in these recordings.

5.2.4.5 Effects of cortisol and noradrenaline

Cortisol and noradrenaline produced patterns similar to that obtained with **natural MS 222** when added to the blood. This observation also stated the possibility of these two hormones being involved in the accelerated and/or

premature clotting or hypercoagulable blood. These results indicated that, under severe stress conditions, both these hormones were released as primary factors, causing the formation of tiny blood clots which may result in the death of those fish species that are very sensitive to stress. The two species used for this study, did not display such mortalities.

5.2.4.6 Effects of adrenaline and noradrenaline

The results obtained with the addition of both adrenaline and noradrenaline to the blood of unstressed fish, were virtually similar to those for cortisol and noradrenaline. These observations vindicated the participation of both catecholamines in causing different clotting patterns that deviated from those recorded during unstressed conditions. **Mazeaud *et al* (1977)** also measured increased levels of catecholamines and corticosteroids during stress, which confirmed the role of both adrenaline and noradrenaline in accelerating clotting or producing hypercoagulable clotting patterns in fish blood. **Smit (1990)** demonstrated that under such conditions, the level of adrenaline was higher than that of noradrenaline. It was possible, that adrenaline may contribute more considerably to the altered **TEG** than noradrenaline through its possible actions on the thrombocytes.

5.2.4.7 Effects of cortisol, adrenaline and noradrenaline

When cortisol, adrenaline and noradrenaline were added in equal quantities to the blood of unstressed fish, similar **TEG** patterns to those recorded for cortisol and noradrenaline, were obtained. These results corresponded to

accelerated or premature clotting, thereby indicating possible **DIC** through thrombocyte aggregation. Such effects may produce mortalities in those fish species that are more sensitive to stress.

5.2.4.8 Effects of cortisol, adrenaline and a low concentration of noradrenaline

When the above combination of stressor hormones was added to the blood of unstressed fish, a typical **TEG** pattern for stressed fish were observed. This observation suggested that all three stressor hormones were involved in accelerated clotting during stress. Noradrenaline, in combination with cortisol and adrenaline, appeared to contribute more significantly to the recorded stress effects. **Mazeaud *et al* (1977)** and **Smit (1990)** recorded significant differences between circulating adrenaline and noradrenaline levels, with adrenaline being significantly higher in the circulating blood of stressed fish. The effects for the hormone combination applied in this case, produced similar **TEG's** to those recorded for stressed fish. During severe stress situations, higher levels of noradrenaline may be secreted which may ultimately produce the same effects as those recorded for high levels of the three hormones with the subsequent formation of lethal clots. It is possible, that, such effects may explain the delayed effects of disseminated intravascular clotting and resultant mortalities after a certain time period. It is, furthermore, essential to consider the individual roles of these hormones on the circulating thrombocytes to produce the results obtained. These can be explained as follows:

The importance of cortisol is connected to its ability to mobilize glucose for energy purposes **(Mazeaud *et al* 1977).** This observation was confirmed by **Hattingh (1976)** when he identified elevated glucose levels in ***Labeo umbratus*** exposed to stress. Such elevated levels for ***Clarias gariepinus, Schilbe (S) mystus depressirostris*** and ***Oreochromis mossambicus*** were also reported by **Smit *et al* (1990).** The latter authors also suggested that elevated stressor hormones may be lethal in those species more sensitive to stress, such as ***Oreochromis mossambicus.*** **Barton & Peter (1982)** also reported that severe longterm stress also produced continued elevated levels of these hormones. The observations recorded in this study suggested that the effects of added artificial hormones to the blood of unstressed fish might have a significant effect on accelerated clotting under stressed conditions, either by direct accelerated clotting of the blood or through its effects on thrombocyte aggregation. The last observation is extremely important, since **Smit (1980)** indicated that stress might result in catecholamine induced contraction of the splenic capsule, whereby large numbers of blood cells, including thrombocytes were added to the circulation. This was confirmed by **Van Vliet (1981)** for ***Cyprinus carpio*** and ***Oreochromis mossambicus.*** The significance of this reaction must be coupled to the mobilization of energy during stress as indicated previously. Thus, increased thrombocyte levels as well as increased circulating levels of **ADP** resulting from energy mobilization and consumption, might contribute to accelerated clotting. The consequences of such elevated stressor hormone levels, may be extremely detrimental. Hypercoagulable blood or disseminated intravascular clotting may obstruct or impede blood flow to poorly perfused organs or vital organs, thereby causing ischemia to tissues and the formation of necrotic tissues. Under extreme stress

circumstances, it may result in the formation of lethal blood clots with ensuing death. Furthermore, it should also be considered that, especially those species that are not sensitive to stress and have the ability to survive adverse conditions, may have a supportive mechanism to aid their survival. One such mechanism appeared to be the existence of a well-developed fibrinolytic system to counteract clot formation by dissolving the clot whilst still in the process of being formed. This suggested that, when stress was terminated, the hypercoagulable state must be reversed or inactivated to avoid death. This was, indeed, confirmed in long standing blood samples, but was not quantified. It, therefore, requires further evaluation to confirm the suggestions made. Notwithstanding, activation of fibrinolysis may produce rapid lysis of clots to reduce mortalities. These observations may explain the greater adapting abilities of these two species under adverse or abnormal aquatic conditions. Furthermore, observations made over many years in the laboratory, suggested that gastric fish have a greater ability to combat stress when compared to agastric fish. Future studies of this nature should contribute more significantly to a better understanding of stress in freshwater fish. Nevertheless, the strength of the stress stimulus as well as the ability of fish to respond to a particular stress is of utmost importance. The suggestions made above include the possibility that certain fish species are better equipped with a sensitive mechanism to respond in a graded manner to prevent possible causes of lethal mortalities.

All the above observations, assure that stress is a complicated phenomenon to which there is no simple definition. It does, however, confirm that stress is not caused by a single stressor hormone acting on a particular cell type,

organ or system, but that all functional parts of the living body or organism, are simultaneously affected to a greater or lesser extent by a combination of stressor hormones of which some are not yet known. It is also possible that other known hormones, whose stressor functions have not yet been elucidated, may also induce stressor effects that are not yet described. Such possibilities should also be considered when evaluating stress in freshwater fish. Furthermore, it also appeared as if each functional unit, organ or system, may not respond in the same magnitude to stress, but rather that each of these are affected differently during any particular stress situation. Such different physiological effects might have been responsible for the numerous definitions of stress, which all remain acceptable. These, however, tend to describe the reactions of individual systems and do not account for the interaction of the various systemic functions. In spite of such conclusions, they, nevertheless, share the same basic principles as discussed earlier. The major response to stress appears to be a total response to overcome the problem experienced. Based on these observations it can be concluded that all stress responses can be classified into different categories. These reflect the neuro-endocrine or primary responses that can be determined by analyzing the levels of the various stressor hormones, such as catecholamines and cortisol, in the fluid systems of the body. These initiate secondary effects or physiological adjustments caused by the intervention of the coordinating systems to a particular environment followed by a tertiary response to counteract the original stressor stimulus. The subsequent physiological adjustments may result in effects such as osmotic and metabolic disturbances which can be measured by analyzing the fluid compartments of the body. Other diagnostic effects that can be measured for

this purpose, include, increased cardiac rates and high blood pressures. Such measurements therefore provide an indication of the ability of a particular fish species to maintain homeostasis. These effects are of significant importance, due to the fact that some species are more susceptible or sensitive to a changing environment than others. These explain the relatively high mortality rates of certain species such as ***Oreochromis mossambicus*** because they are extremely sensitive to stress in laboratory situations. All these reactions, result in a tertiary response whereby the animal as a whole takes evasive action to overcome exposure to a particular stress condition.

Of particular importance was the observation by **Maetz (1974)** involving the mechanism producing osmoregulatory disturbances induced by stress. This particular mechanism affected the gills and the kidneys whereby the mineral balance was totally disturbed. This is especially relevant to aquaculture practices, since water or salt over or underload may produce a marked effect within minutes. A significant relationship had thus been established between neurohumoral and osmotic changes that led to the suggestion by **Maetz (1974)** that alterations in the circulatory levels of adrenaline were primarily responsible for the effect of handling on water balance of the fish. Stress was shown to affect sodium balance in fish and a loss of sodium across the gills was also reported in rainbow trout following exercise **(Randall** *et al* **1972)**. In the freshwater adapted rainbow trout gill, adrenaline was shown to increase sodium uptake **(Richards & Fromm, 1970)**. This was also confirmed by **Payan** *et al* **(1975)**. Thus, all the biological processes involved in stress still remain poorly understood. However, the degree to which fish are affected by the stress of capture and transportation, varied from species to

species. In ***Clarias gariepinus,*** lower levels of stressor hormones were released, which possibly explain the greater ability of the sharptooth catfish to survive adverse conditions **(Smit, 1990)**. All the above information provided sufficient evidence that exposure of fish to stress resulted in the accelerated clotting of blood that appeared to be a function of cortisol, adrenaline and noradrenaline. More important, however, was that this investigation contributed significantly to the possibility that fish may display graded responses to stress. The **TEG** recordings obtained suggest that such graded responses may occur in the two fish species studied and that this may also be true for other species. Such graded responses to stress can be classified as follows when based on the results recorded:

1. **Unstressed fish:** **TEG's** recorded for this group of fish corresponded with results obtained for human blood as well as those for ***Cyprinus carpio*** and ***Oreochromis mossambicus*** **(Van Vliet, 1981)**. The measured parameters for such **TEG's** were in line with those recorded for the standard wine - glass shaped recordings. Such results therefore suggested that immobilization of fish with **neutralized MS 222** and electronarcosis were suitable and effective for obtaining blood samples from unstressed fish.

2. **Chemical stress:** Anaesthetization with natural, acidic **MS 222** produced **TEG'S** that deviated significantly from those obtained with neutralized **MS 222.** Values for the different TEG parameters measured corresponded to those associated with **DIC** in human blood. Visual observation of the remaining blood sample, indicated the presence of minute clots. Similar effects were observed in blood samples acidified with acetic acid. Such

observations were not quantified or qualified, but merely used to confirm the acidic effect on the clotting mechanism of fish blood. These observations suggest possible chemical stress effects, similar to those obtained when adrenaline and noradrenaline were added to the blood of unstressed fish. Similar and more pronounced effects were also recorded when **MS 222** was added to the blood sampled from unstressed fish. Significantly different **ITP** and **TI** values resulted. These confirmed the likelihood of chemical stress resulting primarily from MS 222 related pH effects on the blood as confirmed by **Smit (1980)**. Such reactions may lead to increased circulatory catecholamine levels. Chemical stress therefore appeared to elicit a different neuro-endocrine response in both fish species that are different from those recorded under differing stress conditions.

3. **Handling stress:** Handling of fish for blood sampling also produced a **TEG** recording different to those discussed above. These were also reflected in significantly different **ITP** and **TI** values. In both species these conformed to effects induced by the addition of adrenaline to the blood of unstressed fish. These results coincided with short term unexpected physical stress, implicating the immediate release of adrenaline to initiate physiological responses to accommodate stress effects immediately. It therefore, appeared that the first neuro-endocrine response in all cases initiated the release of adrenaline. The significance of this release is determined by the nature of the stress stimulus and subsequent physiological responses.

4. **Short term and chronic stress** effects produced neuro-endocrine responses

involving the secretion of hormones to combat stress by mobilizing nutrients for energy and to adjust systemic functional activities to overcome the crisis experienced to ensure survival. Furthermore, such neuro-endocrine responses also produced secondary effects which were not recorded during this investigation. In general, the clotting activities observed corresponded to those obtained when adrenaline, cortisol and low levels of noradrenaline were added to the blood of unstressed fish. These were also clearly demarcated in the **ITP** and **TI** values measured. In general, the net effects indicated accelerated clotting, but not to such an extent that **DIC** resulted in lethal mortalities. These observations suggest that the nature of the stress produced a neuro-endocrine response leading to increased cortisol and adrenaline levels with a lesser release effect of noradrenaline.

5. **Longterm acute stress**: The results obtained with such underlying effects, corresponded to those obtained for high levels of cortisol, adrenaline and noradrenaline added to the blood of unstressed fish. Neuro - endocrine responses were related to longterm strategies to assist the different functional systems to cope with the stress situation. Under these circumstances, it is expected that mortalities that can not be avoided by neuro -endocrine reactions may result. Lethal clots or **DIC** may occur, and could impede natural blood flow to poorly perfused tissues or the formation of clots that could block blood supply to vital organs or tissues. Such reactions may explain stress mortalities or the appearance of necrotic patches on the body surface of fish, in particular in those species that are sensitive to stress such as ***Oreochromis mossambicus.***

Other important factors to be considered during stress, include the role of increased thrombocyte numbers associated with increased blood cell counts added to the circulation from blood reservoirs. The latter may result from contraction of the splenic capsule under control of the autonomic nervous system **(Smit 1980).** Similar actions can also be connected to the possible effects of stressor hormones on thrombocyte aggregation. Furthermore, it also appeared that the actions of the stressor hormones were aimed at generating the intrinsic system to the stage where thrombin was being formed. This implied that the kinetic coagulability of fish blood was extremely responsive to stress by inducing dynamic coagulation effects to initiate fibrinogen polymerization. Differences in fibrinogen levels between the two species may contribute significantly to the solidity of clot formation. Clot formation differences will probably also be enhanced by interspecies differences in circulating calcium levels. These observations suggest that the butter catfish was more sensitive to stress effects involving blood coagulation. Nevertheless, the nature of the **TEG** amplitudes recorded, suggest the existence of a well developed fibrinolytic system in the blood of both species. This further implied that during the process of clot formation, lysis of the formed clots was initiated. Thus, when stress was terminated, the hypercoagulable nature of the blood had to be reversed or inactivated to ensure survival of the fish. If not, intravascular coagulation may occur to cause lethal mortalities. Thus, the strength and the nature of the stress stimulus are of great significance, since they provide the animal with a sensitive mechanism to respond in a graded manner to ensure survival.

In conclusion, it should be considered that neuro-endocrine reactions in lower vertebrates, such as fish, are closely linked to environmental changes *via* extremely sensitive sensory organs. It is suggested that the fish species studied belong to a lower order nervous system class where the thalamus dominates most reactions and responses. In this way, immediate reactions resulting from changes detected by their sensory organs, would initiate reflex physiological responses through the thalamus in an attempt to accommodate limited exposure to stress. In general, higher vertebrates have the physiological ability to think and to make voluntary and decisive attempts to survive and overcome stress. It is thus possible that certain fish species may appear to be part of this higher vertebrate class. The above suggestion thus support the clotting changes recorded under the above-mentioned categories. These were induced by multiple changes in the hormonal profiles, characterized by activation of the hypothalamo-hypophyseal-adrenal system to maintain homeostasis and to ensure survival during stress conditions. These changes are marked by mobilization of energy reserves that are accompanied by numerous secondary reactions such as accelerated clotting or hypercoagulable blood in conjunction with certain intrinsic blood characteristics to maintain survival. It should also be noted that the effects recorded refer to coagulation characteristics only. These did not consider other impeded systemic functions or systems such as the immune system. If and when the latter became involved, the susceptibility of the animal to develop a disease will be greatly enhanced. The use of neuro-endocrine blocking agents, depleters or antagonists to avoid laboratory induced stress situations, may prove fruitful for future studies of this nature. Nevertheless, it was apparent that the haemostatic mechanisms of fish responded actively to stress, possibly in the form of DIC. The latter was never seriously considered as a significant contributory effect to explain delayed mortalities in fish exposed to stress. The most significant conclusion drawn from these observations, was that fish blood can be considered as an effective indicator of sublethal stress situations. Such effects require quantification and qualification in future studies of this nature.

5.3 Prothrombin times (PT)

In human coagulation studies, the prothrombin time test is carried out to detect congenital and acquired clotting factor deficiencies for fibrinogen **(Factor** I), prothrombin **(Factor II)** and factors **V, VII** and **X (Biggs 1976).** The basis of this test was established by **Quick (1935)** who provided the first information on the activation of intravascular mechanisms by external factors. The principle of this test rests upon the determination of prothrombin levels in the plasma by the addition of tissue thromboplastin and calcium chloride in optimal concentrations to the blood and thereafter measuring clotting times. Increased **PT** times are therefore indicative of reduced levels of the clotting factors mentioned. Since such tests detect deficiencies of five individual clotting factors, it is usually recommended in addition to the partial thromboplastin time test **(PTT).** The latter is used to identify reduced levels or complete absence of clotting factors, excluding **Factor VII.** Both tests are therefore valuable diagnostic aids to determine clotting abnormalities or clotting factor deficiencies **(Dacie & Lewis, 1984).** Since both tests are relatively simple, they can also be extended fruitfully to other animal species, such as fish, for the determination of normal and abnormal clotting times **(Smit & Schoonbee, 1988). Van Vliet** *et al* **(1985)** reported that the preparation of partial thromboplastin reagent was complicated and it was therefore decided to leave such tests for future investigations. The latter authors, however, continued to investigate prothrombin time tests on ***Oreochromis mossambicus*** and ***Cyprinus carpio***.

It was previously indicated that factors **I, II, V** and **X** are common to both the intrinsic and extrinsic pathways. A deficiency in one or more of these factors will result in the prolongation of prothrombin times. When species specific thromboplastins were used, valuable indications can therefore be obtained regarding these factors mentioned. Such preparations were indeed tested by **Van Vliet *et al* (1985)** who confirmed such species specific effects. **Factor VII,** on the other hand, is concerned with the extrinsic pathway. Thus a deficiency of this factor may also influence the prothrombin times. During this investigation, an attempt was therefore made to determine the usefulness of prothrombin times under standard laboratory conditions in unstressed, acutely stressed and also by simulating chronic stress conditions for the two fish species.

Macnab & Ronald (1965) obtained mean prothrombin times of 12,3 seconds when using human plasma and rabbit brain thromboplastin at a temperature of 37 °C. However, at a lower temperature of 7°C, the prothrombin times recorded for human plasma and rabbit brain thromboplastin were 159 seconds. Their findings were in accordance with the results obtained by **Van Vliet (1981),** indicating a possible temperature liability in the fish coagulation system. However, the prothrombin times of the homologous Cod system, showed an increased clotting speed with rising temperature, being 27 seconds at 0°C and reaching 88 seconds at an optimum temperature of 30°C **(Macnab & Roland, 1965).** No clotting occurred at 37°C, probably due to cod fibrinogen denaturation at that particular temperature.

To validate possible species specific reactions to thromboplastin, ***Clarias***

gariepinus and ***Schilbe (S) mystus depressirostris*** thromboplastins were prepared as described earlier and prothrombin times were recorded with various combinations of butter catfish and sharptooth catfish brain thromboplastins, using the plasma of the two fish species mentioned. No significant intraspecies differences for ***Clarias gariepinus*** plasma and thromboplastins prepared from ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** brains, were recorded at room temperature. Furthermore, the results of this investigation indicated prothrombin times of more than 100 seconds for the two species when employing rabbit brain thromboplastin. These observations suggest that the use of ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** plasma, together with thromboplastins prepared from their own brains, showed significant differences for the results obtained with the use of rabbit brain thromboplastins. This can probably be described to species specificity **(Lewis, 1972)**. It is recommended that for tests of this nature, species specific brain thromboplastins be prepared. However, when the fish were subjected to simulated stressful situations, prothrombin times decreased in all instances with ***Clarias gariepinus*** exhibiting slightly higher values than ***Schilbe (S) mystus depressirostris.*** This observation may be associated with the ability of ***Clarias gariepinus*** to survive adverse aquatic conditions. It does, however, not explain the interspecies differences in reaction times. These may be ascribed to the relatively low level of thromboplastins in the brain of sharptooth catfish or differences in the circulating levels of clotting factors of the two species studied as indicated in **Table 19.** In this study, it was also observed that sampling blood by cardiac puncture in ***Clarias gariepinus,*** increased the incidence of obtaining clotted samples. When sampling blood by venipuncture, this phenomenon did not

occur. The clot formed when applying the cardiac puncture technique, probably occurred as a result of higher tissue and/or tissue fluid thromboplastin levels (excluding the brain) during sampling, since the distance of needle penetration to the heart is significantly longer. Sampling blood by cardiac puncture can not be recommended, since it may coincide with the added sampling of tissues and/or tissue fluid. This clot formation also occurred with the use of excessive levels of anticoagulant. Moreover, this technique virtually excluded the possibility of obtaining atraumatic samples **(Van Vliet 1981**). Absence of the clot when venipuncture is used, may be ascribed to the lesser tissue distance of needle penetration, thereby collecting less tissue or tissue fluid in the blood sample. The latter technique therefore eliminated the possibility of obtaining a traumatic blood sample as explained by **Van Vliet (1981).** It can be concluded that blood sampling by venipuncture is the recommended technique for use in scaleless fish. Penetration of the blood vessel to allow bleeding before sampling with the syringe, was also not effective. Furthermore, when comparing clotting and prothrombin times of the fish species studied with those of human blood, it was observed that fish blood clots slower than human blood. It was also established that the clotting of fish blood was activated more rapidly under stress conditions in both species used for this investigation. This observation supported the **TEG** findings of **Van Vliet** *et al* **(1985)** for ***Oreochromis mossambicus*** and ***Cyprinus carpio***. It therefore appeared that such reactions are similar, but not of the same magnitude, in most fish species, with significant differences in tissue thromboplastin levels in those species that are more sensitive to stress such as ***Oreochromis mossambicus.*** These interspecies activation differences may also be associated with differences in

thrombocyte numbers in the circulation during standard and stressful conditions as suggested by Van **Vliet *et al* (1985).** Apart from differences in thrombocyte numbers, different types of thrombocytes related to shape as well as numbers for each type, have also been observed in numerous freshwater fish species **(Pieterse, 1982; Ellis, 1977; Van Vliet, 1981).** These have to be studied carefully in future investigations of this nature. **Van Vliet *et al* (1985)** also recommended that blood sampled for coagulation purposes, should be obtained from unstressed fish sedated with the use of neutralized **MS 222 (Smit 1980)** or neutralized **Benzocaine hydrochloride (Ferreira *et al* 1979).** The present results also confirmed species specificity in brain thromboplastins, which makes rabbit brain thromboplastin not suitable for use with fish blood. It is therefore emphasized that species specific substances and reagents must be used when investigating coagulation strategies for different fish species under standard laboratory and stress conditions.

The above information provides adequate and sufficient evidence that fish blood contained at least factors I, II, III, V, VII and X. Thus, it may be concluded that the clotting process in fish was similar to that of higher vertebrates. These results served as a basis for determining the presence of clotting factors in the circulating blood of fish and to identify their role during stress.

5.4 Blood clotting factors in freshwater fish.

As explained in **Chapter 2,** the reactions induced by clotting factors responsible for the coagulation of blood, are divided into two major

pathways, the intrinsic and the extrinsic mechanisms (**Fig. 3**). Factors **XII, XI, IX, VIII, X, V** and prothrombin participate in the intrinsic pathway whereas tissue thromboplastin, factors **VII, X, V,** and prothrombin participate in the extrinsic pathway. These two pathways connect when **factor X** is activated. Thus, **factor X** plays a central role in the coagulation process, since it is activated independently in either the intrinsic or the extrinsic pathway. It is also a clinical observation that a defect in either system can give rise to hemorrhagic symptoms and that both systems must be intact for normal haemostasis to occur **(Dacie & Lewis, 1984).**

Studies on blood coagulation of lower vertebrates, have revealed differences in clotting power from various groups of fish. Elasmobranchs were reported to be essentially hemorrhagic, containing very little or no prothrombin in their circulation **(Lewis, 1972)**. Teleost fish, on the other hand, have a rapidly blood clotting system **(Doolittle & Surgenor, 1962)**. **Smit & Schoonbee (1988)** also recorded differences in clotting times for traumatic and atraumatic blood samples, as well as in the clotting times for the intrinsic and extrinsic coagulation pathways. This evidence was also supported by **Van Vliet *et al* (1985)** when employing the thrombelastograph for coagulation studies. Although factors attributable to species specificity warrant human routine clotting screening tests for fish blood unacceptable, they can be used as a useful point of departure to determine clotting activities of fish blood. It should, however, be remembered that they do not provide a true reflection of actual intrinsic and extrinsic clotting activity. The results obtained with the testing procedures used in this study, support and confirm the presence of all clotting factors in the blood of fish, being similar to those for human blood.

Smit & Schoonbee (1988) observed that the extrinsic factor clotting times were almost half those obtained for the intrinsic times, thus corresponding with the outline given by **Guyton (1986)** for human blood as well as those recorded in this study.

In order to fully understand the process of coagulation under stress conditions, it was essential to evaluate the different roles of these factors under such conditions.

The results obtained in this investigation, support earlier evidence, that clotting times for the different factors in the intrinsic pathway were significantly longer ($p \leq 0.001$) than the values recorded for the extrinsic factors in both ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris.*** Clotting times for the intrinsic pathway in ***Clarias gariepinus*** were higher than those for ***Schilbe (S) mystus depressirostris*** when exposed to both chronic and acute stress. Similar results were observed for the extrinsic pathway in both species. This observation is probably indicative of the ability of ***Clarias gariepinus*** to survive adverse aquatic conditions. Furthermore, results recorded for fish during acute and chronic stress, suggest that these factors were consumed to confirm the theory of hypercoagulable blood or accelerated clotting under stressful conditions. This increased the possibility of **DIC** occurring during stress. The above information therefore provided sufficient evidence for the presence of a well developed coagulation system in the blood of the two fish species studied. Furthermore, evidence was also provided to support the suggestion that, under severe stress situations, the intrinsic factors were first activated by stressor hormones to accelerate the

clotting process. These, in turn, may be responsible for the development of **DIC** in the blood of most freshwater fish species. The magnitude of the **DIC** may be ascribed to interspecies differences in the tissue levels of thromboplastin, clotting factor levels, normal and stressed stressor hormone levels and a number of other, as yet unknown factors. It should also be noted that the activation of the intrinsic coagulation system, resulted in the intravascular conversion of plasma to serum with the production of disseminated minute fibrin fragments. When this course of events is allowed to continue, as it happens under severe stressful conditions, there is transformation of plasma from the liquid phase to a solid phase, and death obviously ensues.

6. Thrombocytes aggregation studies.

Reports on aggregatory reactions of fish thrombocytes are relatively scarce. Of significant interest, however, was that several reports referred to fish thrombocytes as the clotting cells of blood **(Doolittle & Surgenor,** 1962; **Belamarach *et al* 1968; Ellis, 1977).** These did not describe the qualitative and quantitative actions of these cells during aggregation. Investigations into the physiological effects of rapid decompression on the haemostatic mechanism in trout, revealed that circulating thrombocyte levels changed quickly in response to small perturbations in gas or hydrostatic pressures **(Casillas *et al* 1976). Kayama *et al* (1985, 1986 & 1987)** studied prostaglandin synthesis and aggregation behavior of carp and rainbow trout thrombocytes in comparison with human platelets. Their observations suggested that prostaglandins were responsible for a reversible aggregatory

response. **Woodward *et al* (1981)** reported that the platelet factor release reaction in mammalian platelets, was usually preceded by a change in shape whereby they became more rounded, extended pseudopodia and aggregated loosely. At low concentrations of **ADP or 5-hydroxytryptamine (5-HT),** the platelets did not release sufficient aggregatory components and subsequently dissociated from each other and returned to their normal shapes. The nature and origin of **ADP** becoming available for aggregation, is not well established. **Kayama *et al* (1987)** suggested that **ADP** in humans may be derived from the injured tissues, erythrocytes or from the platelets. The latter authors also pointed out the presence of other circulating aggregating agents such as thrombin and adrenaline which may induce the release of endogenous **ADP** from the platelets. Particulate material such as collagen, antigen-antibody complexes, serotonin and **AHA** also have this ability.

In this chapter, it was indicated that the stress response included mobilization of energy reserves whereby **ATP** was converted to **ADP,** either inside the cells or in the circulating blood. Thus, two sources may therefore provide the necessary **ADP** to initiate aggregation of thrombocytes in the circulatory system. If the aggregatory stimulus was strong enough, release of thrombocyte aggregatory factors may occur. This release coupled to the change in thrombocyte shape, allowed the thrombocytes to form a firm mass of cells through the activation of the intrinsic clotting mechanism or trauma at the site of vascular injury. Such actions therefore possibly provided the necessary foundation for fibrin deposition and further clot solidification **(Harker, 1974).** The latter author suggested that trout thrombocytes were aggregated by various agents and retained their rod shapes, especially at low

concentrations of these agents. At higher concentrations, there was a tendency for rod shaped thrombocytes to be less, and rounded forms being more dominant **(Woodward *et al* 1981). Stiller *et al*** (1974) reported that bullfrog thrombocytes, which were previously found to be unaffected by **ADP,** were strongly aggregated by **ATP (500-200 μM),** but not by **GTP** (guanosine triphosphate). A significant, but less dramatic, aggregation of duck thrombocytes by **ATP** was also shown **(McFarlane & Mills, 1975).** Although several reports implicated thrombocytes as the important clotting cells of fish blood **(Doolittle & Surgenor, 1962; Belamarich *et al* 1966, 1968),** very few specified the role and behavior of thrombocytes of fish blood during adverse conditions.

In this investigation, it was essential to observe the aggregatory behavior of thrombocytes in the two fish species before assessing their effects during stress. The most likely reason for the low research output on thrombocyte aggregation studies in freshwater fish, may be connected to the problems experienced in preparing thrombocyte rich plasma. With human blood, it is a fairly simple action, since platelet rich plasma **(TRP)** can be obtained by centrifuging blood at low speeds **(Dacie & Lewis, 1984).** Platelets have a low suspending action that are responsible for their retention in the supernatant. On the other hand, fish thrombocytes are true cells as explained previously. These settle with the other suspended cells to make **TRP** preparation more difficult. In this investigation, the whole blood aggregometer was more accessible to investigate and report on thrombocyte aggregation for the two species studied.

A report by **Belamarch *et al* (1966)** suggested that **ADP** is not a suitable aggregatory reagent for non-mammalian vertebrates, since they could not detect adequate aggregatory responses in their studies on fish blood. This conclusion was premature, since **ADP** strongly aggregated ***Clarias gariepinus*** and ***Schilbe (S) mystus depressirostris*** thrombocytes. Furthermore, when **ADP** was used at different concentrations for aggregation of fish blood in the two species studied, significantly different responses were recorded. Higher concentrations of **ADP** produced 82% aggregation, whereas lower concentrations showed a decreased aggregation percentage. These results therefore supported the findings of **Woodward *et al* (1981),** that **ADP** aggregated fish thrombocytes in a dose dependent manner. The latter author also suggested that **ATP,** at identical concentrations to **ADP,** also strongly aggregated trout thrombocytes and that the percentage aggregation produced by **ADP** and **ATP** was only significantly different ($p \leq 0.05$) at 1 **μM**. As previously explained, the significance of energy mobilization during stress as a result of neuro-endocrine responses with resultant **ADP** availability, may contribute significantly to the aggregatory behavior of the thrombocytes in the fish species studied. It was also suggested by **Van Vliet (1981),** that stress produced an approximate 30% increase in thrombocyte levels. Stressor hormones therefore play a dual role by increasing thrombocyte numbers through splenic release as well as contributing to the increased **ADP** levels or other aggregating agents to promote possible **DIC.**

The significance of thrombocyte aggregation agents in studies of this nature, can only be explained in terms of information available on similar studies with human platelets as well as those for other vertebrates. Aggregation of human platelets is induced by **thromboxane A_2 (TXA_2),** which is produced from

arachidonic acid (AHA) (Hamberg *et al* 1975). Kayama *et al* (1986) reported on the conversion of **AHA** to **prostaglandins (PG)** in carp and rainbow trout thrombocytes. Furthermore, the conversion from arachidonic acid to a different type of thromboxane, was detected in both carp and rainbow trout thrombocytes. On the other hand, **Matsumoto *et al* (1989),** referred to the significance of **AHA** in producing prostaglandins that can reverse thrombocyte aggregation. These findings were based on the addition of certain specific prostaglandins to thrombocyte aggregates. The significance of such conversions are not clearly understood, although these findings suggest that such agents may also be involved in preventing or reversing the aggregatory reactions of fish thrombocytes. Thus, aggregatory agents may have dual actions, that is, possibility of thrombocyte inducing aggregation as well as reversing the process. Nevertheless, the magnitude of the changes, suggest a common mechanism for thrombocyte aggregation that may be dependant on the strength of the stress stimulus. The nature and scope of the present investigation could not detect such reversible actions with the use of a whole blood aggregometer. Furthermore, interspecies differences based on shape and size of individual thrombocyte types and numbers as well as total thrombocyte counts, may also play a significant role in evaluating such effects. The exact mechanism for such responses therefore require further investigation.

In this investigation, when **AHA** was used as an aggregating agent for sharptooth catfish and butter catfish thrombocytes, it was observed that **AHA** produced dose - dependant aggregation responses in both fish species. The above results therefore suggest that **AHA** induced thrombocyte

aggregation is similar to **ADP**. When decosahexonoate **(DHA)** was used, no aggregation was observed in both fish species. **Matsumoto *et al* (1989)** did not detect the synthesis of prostaglandins from **DHA** in the four fish species they studied. This observation therefore indicated that the conversion of an aggregating agent to prostaglandins is the primary determining factor for the successful use of any chemical agent as an aggregatory substance in order to reverse aggregatory reactions. Therefore, it can be concluded that **DHA** cannot be used as an aggregating agent for fish blood in general.

Woodward *et al* (1981) discovered that epinephrine aggregated trout thrombocytes. Epinephrine also aggregate mammalian platelets **(Mills & Roberts, 1967)**, but no other non-mammalian vertebrate tested, exhibited a similar response. In fish, as in man, stress elevated circulating epinephrine levels **(Mazeaud *et al* 1977; Turner & Bagnera, 1971). Nakano & Tomlinson (1967)** reported plasma epinephrine levels in stressed rainbow trout, varying between 1.0 -2.0 **μM**, falling within the range of ***in vitro*** aggregation of trout thrombocytes. **Cassillas (1978)** reported increased thrombocytes counts along with elevated plasma epinephrine levels following compression stress in rainbow trout. This correlated with the findings of **Cassillas & Smith (1977)** and also earlier findings of this investigation, that the whole blood clotting times of fish was significantly reduced following stress. Preliminary investigations suggested, that when epinephrine was used as an aggregating agent with the thrombocytes of unstressed butter catfish and sharptooth catfish, aggregatory responses that occurred depended on the concentration of epinephrine used. When similar concentrations of epinephrine were used for thrombocytes of stressed fish, no responses were recorded, or negative

aggregation results were obtained. Presumably, this was partly due to the increase in circulating thrombocytes and increased aggregation of these thrombocytes in the presence of epinephrine released during stress. In contrast to these disadvantageous effects of epinephrine on the fish clotting system, severe stress might elevate circulating epinephrine levels to such an extent that it may result in widespread intravascular clotting and subsequent death of the fish. **Smit (1980)** indicated that the stress response resulted in the contraction of the splenic capsule whereby large numbers of blood cells were added to the circulation. This author also emphasized the significance of the spleen as a blood reservoir, controlled by the sympathetic division of the autonomic nervous system. It is therefore suggested that stress stimulated the sympathetic nervous system, which in turn, produced splenic capsule contraction. Simultaneously, circulating levels of catecholamines also increased. Such observations were also confirmed by **Nakano & Tomlinson (1967), Turner & Bagnera (1971)** and **Casillas (1978).** The blood cells added to the circulation, may therefore be associated with the increased thrombocyte counts. The latter was observed by several authors **(Van Vliet 1981; Woodward *et al* 1981; Fujikata & Ikeda 1985)** for different fish species. The causative effects of epinephrine and other stressor hormones as stimulating agents for accelerated clotting of fish blood, have been confirmed with the use of thrombelastography in this investigation. This observation was also supported by **Woodward *et al* (1981)** and confirmed in this study, that adrenaline aggregated trout thrombocytes significantly. It was therefore natural to suggest that the aggregatory and clotting responses measured in this study, confirmed that whole blood clotting times as well as accelerated clotting ***in vitro,*** were reduced by adrenaline. Under stress conditions, this

reaction was augmented by increased circulatory thrombocyte levels. Both factors contributed to increased aggregatory responses with subsequent accelerated clotting. Under extreme stress conditions, these two factors may result in widespread intravascular clotting followed by death of the fish. These observations also corresponded to findings in the laboratory that certain fish species are extremely sensitive to stress. Interspecies differences to various stress situations may therefore result from the number of thrombocytes in the circulation and the level of circulating stressor hormones in relation to the mass and size of the fish. Further studies regarding the role of thrombocytes under stress situations and their interaction with the cellular and enzyme components of the coagulation system, are essential to elucidate the existing problems identified during stress, especially heavy mortalities following capture, transport and handling stress.

7. CONCLUSION

This study provided evidence that blood coagulation in freshwater fish is fundamentally similar to that of higher vertebrates. The use of **neutralized MS 222 (Sandoz)** and electronarcosis are recommended for immobilization of freshwater fish. In contrast, **natural acidic MS 222** was found unsuitable for use with fish blood, since it induced premature clotting which gave false results that cannot be used to explain clotting variations. When fish were exposed to stress situations, such as capture and handling, their clotting systems were accelerated. Furthermore, this accelerated clotting was a function of cortisol, adrenaline and noradrenaline. This corresponded with the observations recorded by **Smit *et al* (1990)**, who indicated that stress was

associated with high levels of cortisol, adrenaline and low levels of noradrenaline. Serious stress effects may result in the secretion of higher levels of noradrenaline. These, in turn, may produce mortalities, resulting from delayed blood coagulation with subsequent death. The role of other adrenal steroid hormones have not been considered and should be evaluated in future investigations of this nature.

Aggregatory studies involved limited investigations to determine whether such actions do occur in indigenous fish species. This was confirmed with the aggregating agents tested for this purpose. Adenosine diphosphate and arachidonic acid caused ***in vitro*** aggregatory responses in fish thrombocytes in a dose dependent manner. These actions provided a sound indication that the fish species studied, had a sensitive and rapid system for initiating haemostasis when necessary. Their exact role under stress situations should also be evaluated in future.

It is important to realize that clot formation ***in vivo*** is a dynamic process, involving interaction between the cellular and enzyme components of the clotting mechanism that was found to be similar to those of higher vertebrates. The results recorded, clearly suggested the role of stressor hormones in accelerating coagulation when these animals were stressed. It should be emphasized, however, that blood coagulation merely presents a single functional system in the interaction of many systems in the body. Caution should therefore be taken not to consider the effects of stress on individual systems only, but also to understand the interaction between the various functional systems to explain the phenomenon of biological stress.

Since the animals used in this study belonged to a lower order of vertebrates, it should be understood that their responses to stress might differ from those of higher vertebrates.

In general, the results obtained in this study, agreed principally with the concepts of biological stress outlined by **Mazeaud *et al* (1977).**

CHAPTER VI

RECOMMENDATIONS

CHAPTER VI RECOMMENDATIONS

The results obtained in this investigation provided sufficient evidence that stress affected the blood coagulation mechanism through acceleration of the clotting process. These introductory studies require further investigations into blood coagulation in general with particular reference to the following:

° Venipuncture was the most convenient and effective means for blood sampling in fish without scales, such as ***Clarias*** and ***Schilbe.*** Suitable alternative means for improved blood sampling methods in fish with scales should be investigated.

° This study confirmed the usefulness of thrombelastography as a diagnostic tool to evaluate blood coagulation in freshwater fish. Future studies of this nature should include the determination of known stressor hormone levels and other neuro-transmitters in the blood of stressed and unstressed fish to gain a more accurate measure of their role during clotting. Although only visual observations of blood samples treated with acid were made, more qualitative and quantitative methods should be employed to test this hypothesis.

° Species specific brain thromboplastins were more effective than commercial preparations for determining prothrombin times. These were not used for determining the clotting times of the different coagulation factors involved in the intrinsic and extrinsic pathways. Future investigations should include studies to confirm their nature and role in the coagulation system.

° Qualitative and quantitative methods should be used to determine the thromboplastin levels in different tissues of the fish. This will assist greatly in avoiding clotting of blood during sampling and to determine the most effective sight for the sampling of blood.

° No attempts were made to determine temperature lability of fish thromboplastins and the different clotting factors. These may contribute significantly to a better understanding of the haemostatic mechanism in fish.

° The role of fibrinogen in clot formation, clot retraction and lysis of blood clots was not determined. Fibrinogen and fibrin temperature lability need to be tested and quantified if possible. Furthermore, the effects of stress on fibrinogen were not evaluated.

° Total blood calcium levels for the two species studied were known from previous investigations. These served as a basis for recalcification of the blood. Although it is generally accepted that ionized calcium is essential for blood coagulation, it was not determined and should therefore be included in future investigations of this nature.

° Analysis of clotting factors were undertaken with biochemical test combinations prepared for human blood. Although these were indicative of the presence of clotting factors, valid and reliable techniques should be developed to determine Accurate values for such factors. In this way, a more desirable method for testing stress effects on fish blood can be

determined.

° Arteriosclerosis is a well known disease in higher vertebrates. No such studies for freshwater fish are known. These may provide interesting information with reference to age and diet of different fish species that may be useful for clinical treatment and correction of this disease in higher vertebrates.

All the above information should ensure a significant contribution toward a better understanding of disseminated intravascular coagulation in freshwater fish during stress.

° Thrombocyte studies and aggregation responses in future investigations should include some of the following aspects:

1. Scanning electron microscopy of clot formation with emphasis on thrombocyte shapes and sizes prior and during the clotting process. Such studies should be undertaken on blood samples obtained from stressed and unstressed fish.

2. Thrombocyte counts in stressed and unstressed fish with emphasis on the numbers of the different types as well as their ratio to other blood cellular elements.

3. General thrombocyte biochemistry with reference to release of functional factors during clot formation.

4. Evaluation of suitable means for preparing thrombocyte rich plasma for aggregation studies and to investigate the following:
   a. Effects of natural **acidic MS 222** on aggregation.
   b. Effects of acidity on fish thrombocyte aggregation.
   c. Thrombocyte aggregation inducing or inhibition agents with emphasis on the effects of:
      i. GTP
      ii. Neurotransmitters including acetylcholine
      iii. Other stressor hormones
      iv. Vascular injury and effects of collagen
      v. Inhibitory aggregation agents and their significance with specific reference to blocking agents, depletors and antagonists.

The above suggested programme for future investigations indicate the many unexplored areas existing on the haemostatic mechanism of fish blood. All these activities need to be related to fish mass and size. Furthermore, it was also suggested that laboratory observations implicated marked differences in stress responses between gastric and agastric fish. Finally, the role of other fish functional systems during stress should also be considered when evaluating stress. This is essential, since survival depends on the interaction of the different functional systems when exposed to stress in their natural aquatic environments.

CHAPTER VII

TABLES AND FIGURES

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A. FIGURES.

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B. TABLES

ABBREVIATIONS FOR TABLES 1 - 17

THROMBELASTOGRAPHY

r	=	Clotting time or rate of Thromboplastin generation
k	=	Time or rate at which a clot of certain solidity is formed or a measure of the rapidity of fibrin formation.
r + k	=	Total clotting time
Ma	=	Maximum amplitude of TEG
mm	=	millimeters
ITP	=	Index of Thrombodynamic Potential
TI	=	Thrombelastographic Index

TABLES

The effects of different treatments on TEG recordings of the blood in *Clarias gariepinus* and *Schilbe (s) mystus depressirostris*

TABLE 1

	Neutralized MS 222					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	**MEAN**	**SD**	**RANGE**	**MEAN**	**SD**	**RANGE**
r - mm	12,01	0,12	11,80 - 12,20	12,47	0,07	12,40 -12,60
k - mm	8,13	0,11	8,00 - 8,30	10,18	0,13	10,00 -10,40
ma-mm	65,29	9,17	65,00 - 65,60	70,17	0,12	70,00 -70,40
I.T.P.	22,94	0,32	22,40 - 23,30	22,92	0,29	22,40 -23,30
r + k	20,14	0,17	19,80 - 20,40	22,65	0,17	22,50 -22,90
T.I.	30,84	0,22	30,40 - 31,40	32,27	0,24	31,90 -32,60

TABLE 2

	Electronarcosis					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA METER	**MEAN**	**SD**	**RANGE**	**MEAN**	**SD**	**RANGE**
r - mm	11,67	0,15	11,50 - 11.90	11,98	0,11	11,80 -12,20
k - mm	7,84	0,08	7,70 - 8,00	8,12	0,19	8,00 - 6,60
ma-mm	68.47	0.13	68,30 - 68,80	69,06	0,18	68,80 -69,40
I.T.P.	26,95	0,21	26,50 - 27,10	27,21	0,59	25,80 -27,75
r + k	19,51	0,14	19,20 - 19,70	20.10	0,24	19,90 -20,60
T.I.	28,49	0,22	28,00 - 28,80	29,10	0,36	28,70 -29,81

The effects of different treatments on blood coagulation in *Clarias gariepinus* and *Schilbe (S) mystus depressirostris*

TABLE 3

	Natural MS 222					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	10,11	0,80	10,00 - 10,20	16,27	0,14	16,00 - 16,50
k - mm	14,13	0,13	14,00 - 14,30	20,33	0,37	19,90 - 21,20
ma-mm	50,21	0,09	50,00 - 50,40	41,50	0,62	40,50 - 42,10
I.T.P.	7,76	0,19	7,20 - 7,90	3,31	0,04	3,20 - 3,34
r + k	24,30	0,19	24,00 - 24,50	36,60	0,49	36,10 - 37,60
T.I.	48,20	0,42	47, 70 - 48,70	88,20	1,32	86,40 - 90,20

TABLE 4

	MS 222 added to the blood					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	6,06	0,70	6,00-6,20	6,17	0,16	5,00- 5,50
k - mm	-	-	-	-	-	-
ma-mm	6,55	0,11	6,60-6,80	20,09	0,99	20,00-20,30
I.T.P.	-	-	-	-	-	-
r + k	-	-	-	-	-	-
T.I.	-	-	-	-	-	-

The effects of different treatments on blood coagulation in *Clarias gariepnus* and *Schilbe (S) mystus depressirostris*

TABLE 5

	Neutralized MS 222 without TSC added to blood					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	7.16	0.16	7.00 - 7.40	7.56	0.07	7.50 - 7.70
k - mm	5.72	0.15	5.50 - 5.90	6.47	0.10	6.30 - 6.60
ma-mm	86.33	0.36	85.80 - 87.00	76.39	0.16	76.20 -76.70
I.T.P.	107.72	2.37	104.10 -111.60	50.36	0.72	49.40 -51.70
r + k	12.88	0.25	12.50 - 13.30	14.03	0.13	13.90 -14.30
T.I.	14.91	0.28	14.50 - 15.47	18.36	0.16	18.10 -18.64

TABLE 6

	Natural MS 222 without TSC added to the blood					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	2.00	0.13	1.80 - 2.20	2.07	0.10	2.00 - 2.20
k - mm	1.87	0.07	1.80 - 2.00	1.65	0.12	1.50 - 1.80
ma-mm	115.56	0.28	115.20 -116.00	120.10	0.12	120.00 -120.40
I.T.P.	5300.20	189.30	4950.00-5500.00	6151.10	515.10	5500.00-6600.00
r + k	3.87	0.16	3.60 - 4.20	3.72	0.14	3.50 - 4.00
T.I.	3.35	0.14	3.12 - 3.64	3.10	0.12	2.90 - 3.32

The effects of different treatments on blood coagulation in *Clarias gariepinus* and *Schilbe (S) mystus depressirostris*

TABLE 7

	Stressed fish without the use of TSC					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	**MEAN**	**SD**	**RANGE**	**MEAN**	**SD**	**RANGE**
r - mm	2.11	0.13	1.90 - 2.30	2.04	0.10	2.00-2.10
k - mm	1.82	0.08	1.70- 1.90	1.79	0.07	1.60-1.90
ma-mm	110.10	0.09	110.00- 110.20	100.16	0.05	100.10-100.20
I.T.P.	5448.90	240.00	5210.00-5823.50	5536.02	223.22	5210.00-5823.00
r + k	3.93	0.14	3.70- 4.20	3.83	0.11	3.70-4.00
T.I.	3.56	0.13	3.64- 3.81	3.82	0.11	3.69-3.99

TABLE 8

	Acutely stressed					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	**MEAN**	**SD**	**RANGE**	**MEAN**	**SD**	**RANGE**
r - mm	4,08	0,08	4,00 - 4,20	4,16	0,10	4,00 - 4,30
k - mm	2,45	0,05	2,40 - 2,50	2,42	0,08	2,30 - 2,50
ma-mm	86,05	0,34	85,10 - 86,07	86,07	0,38	85,10 - 86,40
I.T.P.	250,70	5,38	245,60 -255,80	253,90	8,35	245,60 -266,90
r + k	6,53	0,11	6,40 - 6,70	6,58	0,14	6,40 - 6,80
T.I.	7,59	0,12	7,42 - 7,77	7,65	0,16	7,40 - 7,89

The effects of different treatments on blood coagulation in *Clarias gariepinus* and *Schilbe (S) mystus depressirostris*

TABLE 9

	Chronically stressed fish					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	4,07	0,23	3,80 - 4.50	3,94	0,12	3,80 - 4,20
k - mm	2,51	0,24	2,00 - 3,30	2,39	0,11	2,20 - 2,50
ma-mm	80,50	0,71	80,00 - 82,30	80,15	1,03	78,20 - 82,10
I.T.P.	160,75	16,42	133,30 -200,00	158,03	7,38	150,80 -171,30
r + k	6,58	0,33	5,90 - 7,00	6,33	0,12	6,20 - 6,50
T.I.	8,17	0,44	7,24 - 8,75	7,90	0,13	7,72 - 8,13

TABLE 10

	Effects of cortisol					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	20,62	0,64	20,10 - 22,10	22,44	0,23	22,00 - 22,86
k - mm	26,42	0,08	26,30 - 26,50	28,59	0,23	28,20 - 28,90
ma-mm	44,87	0,95	43,60 - 46,50	44,44	0,85	43,20 - 46,20
I.T.P.	2,95	0,01	2,90 - 3,00	2,79	0,04	2,69 - 2,80
r + k	47,04	0,61	46,40 - 48,40	51,03	0,31	50,40 - 51,40
T.I.	104,80	2,96	100,60 -106,60	114,87	2,47	110,30 -118,90

The effects of different treatments on blood coagulation in *Clarias gariepinus* and *Schilbe (S) mystus depressirostris*

TABLE 11

	Effects of adrenalin					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	6,02	0,18	6,00 - 6,50	8,41	0,21	8,20 - 8,90
k - mm	5,49	0,12	5,30 - 5,70	6,87	0,15	6,60 - 7,10
ma-mm	63,22	1,29	62,30 - 66,40	58,51	0,16	58,20 - 58,70
I.T.P.	29,65	0,64	28,50 - 30,70	21,04	0,58	20,10 - 21,80
r + k	11,72	0,22	11,40 - 12,00	15,28	0,28	15,10 - 15,80
T.I.	18,54	0,44	17,90 - 19,20	26,11	0,40	25,70 - 26,90

TABLE 12

	Effects of Noradrenalin					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	11,40	0,31	10,80 - 11,80	10,60	0,18	10,40 - 11,00
k - mm	7,53	0,27	6,90 - 7,80	7,19	0,25	7,00 - 7,80
ma-mm	72,18	1,06	70,40 - 74,20	72,42	0,31	72,00 - 73,10
I.T.P.	33,85	0,70	32,90 - 34,70	35,93	1,15	32,90 - 36,70
r + k	18,93	0,46	18,30 - 19,60	17,79	0,33	17,50 - 18,60
T.I.	26,23	0,72	25,21 - 27,68	24,56	0,44	24,10 - 25,67

The effects of different treatments on blood coagulation in *Clarias gariepinus* and *Schilbe (S) mystus depressirostris*

TABLE 13

	Effects of Adrenaline and Cortisol					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	1,75	0,95	1,60- 1,90	4,13	0,14	4,00- 4,40
k - mm	0,85	0,17	0,60- 1,10	2,00	0,15	1,80- 2,20
ma-mm	98,53	0,13	98,40- 98,80	100,20	0,13	100,00- 100,40
I.T.P.	5985,10	1284,90	4454,00-8166,00	4974,80	327,20	4500,00-5500,00
r + k	2,60	0,19	2,30- 2,90	6,13	0,13	5,90- 6,30
T.I.	2,64	0,20	2,32- 2,94	6,12	0,12	5,88- 6,29

TABLE 14

	Effects of Cortisol and Noradrenaline					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	MEAN	SD	RANGE	MEAN	SD	RANGE
r - mm	20,25	0,17	20,00 - 20,50	22,38	0,21	22,00 - 22,80
k - mm	40,55	0,05	40,50 - 40,60	36,49	0,17	36,20 - 36,80
ma - mm	38,28	0,50	36,90 - 38,60	40,41	0,17	40,00 - 40,60
I.T.P.	1,51	0,01	1,50 - 1,52	1,84	0,01	1,82 - 1,85
r + k	60,80	0,19	60,50 - 61,10	58,87	0,32	58,40 - 59,40
T.I.	158,70	2,13	156,70 -164,40	145,68	1,05	144,50 -148,00

The effects of different treatments on blood coagulation in *Clarias gariepinus* and *Schilbe (S) mystus depressirostris*

TABLE 15

	Effects of Adrenaline and Noradrenaline					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	**MEAN**	**SD**	**RANGE**	**MEAN**	**SD**	**RANGE**
r - mm	10,25	0,47	9,60 - 10,80	13,32	0,31	13,00 - 14,00
k - mm	18,38	0,19	18,00 - 18,60	25,87	0,74	24,50 - 26,50
ma - mm	50,45	0,18	50,20 - 50,80	43,64	1,08	42,50 - 45,80
I.T.P.	5,43	0,05	5,30 - 5,50	3,29	0,10	3,09 - 3.48
r + k	28,63	0,44	27,90 - 29,10	39,19	0,90	37,50 - 40,50
T.I.	56,75	0,93	55,20 - 57,80	89,84	2,79	85,60 - 93,80

TABLE 16

	Effects of Adrenaline, Noradrenaline and Cortisol					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	**MEAN**	**SD**	**RANGE**	**MEAN**	**SD**	**RANGE**
r - mm	16,38	0,34	16,00 - 17,00	15,04	0,40	14,30 - 15,60
k - mm	-	-	-	-	-	-
ma - mm	24,37	0,23	24,00 - 24,80	30,20	0,97	28,50 - 32,40
I.T.P.	-	-	-	-	-	-
r + k	-	-	-	-	-	-
T.I.	-	-	-	-	-	-

TABLE 17

	Effects of Adrenaline, Cortisol and low concs. of Noradrenaline					
	Clarias gariepinus			*S(s) mystus depressirostris*		
PARA-METER	**MEAN**	**SD**	**RANGE**	**MEAN**	**SD**	**RANGE**
r - mm	4,11	0,07	4,00 - 4,20	3,64	0,18	3,40 - 3,90
k - mm	2,46	0,16	2,30 - 2,80	2,08	0,11	1,90 - 2,30
ma - mm	82,70	0,90	81,40 - 84,20	88,60	0,22	88,30 - 89,00
I.T.P.	189,64	9,34	168,20 -197,80	317,13	19,98	334,70 -405,20
r + k	6,57	0,17	6,40 - 6,90	5,72	0,22	5,30 - 6,00
T.I.	7,95	0,25	7,60 - 8,38	6,46	0,25	5,98 - 6,79

Abbreviations used for Tables 18,19,20 and 21

A = Neutralized MS 222

B = Electronarcosis

C = Natural MS 222

D = Effects of Natural MS 222 added to blood

E = Neutralized MS 222 without the use of TSC

F = Natural MS 222 without the use of TSC

G = Effects of stress without TSC added

H = Acutely stressed fish

I = Chronically stressed fish

J = Effect of cortisol (C)

K = Effect of adrenaline (A)

L = Effect of noradrenaline (NA)

M = Effect of A and NA

N = Effect of A and C

O = Effect of C and NA

P = Effect of A, C and NA

Q = Effect of A, C and 1\2 NA

Table 18: Mean bloodcoagulation variables (TEG) recorded for different treatment groups in *Clarias gariepinus*.

Treat-ments	r-mm times	r-time	k-mm	k-time	r+k times	Ma-mm	ITP	TI
A	12.01	6.01	8.13	4.07	10.07	65.29	22.94	30.84
B	11.67	5.84	7.84	3.92	9.767	68.47	26.96	28.49
C	10.01	5.05	14.13	7.07	12.12	50.21	7.75	48.20
D	7.16	3.58	5.72	2.86	6.44	86.33	107.72	14.91
E	2.11	1.05	1.82	0.91	1.96	110.10	5448.90	3.56
F	2.00	1.00	1.87	0.94	1.94	115.56	5300.20	3.85
G	6.06	3.03	-	-	3.03	6.55	-	-
H	4.08	2.04	2.45	1.23	3.27	86.05	250.70	7.59
I	4.07	2.04	2.51	1.26	3.29	80.50	160.75	8.17
J	20.62	10.31	26.62	13.31	33.93	44.87	2.95	104.80
K	6.02	3.01	5.49	2.57	5.76	63.22	29.65	18.54
L	11.40	5.70	7.53	3.77	9.47	72.18	33.85	26.23
M	10.25	5.13	18.38	9.19	14.32	50.45	5.43	56.75
N	1.75	0.87	0.85	0.43	1.29	98.53	5985.10	2.64
O	20.25	10.13	40.55	20.27	30.40	38.29	38.29	60.80
P	16.38	8.19	-	-	8.19	24.37	-	-
Q	4.11	2.06	2.46	1.23	5.35	82.64	189.64	7.95

Table 19: Mean bloodcoagulation variables (TEG) recorded for different treatment groups in *Schilbe (S) mystus depressirosris*

Treat-ments	r-mm times	r-time	k-mm	k-time	r+k times	Ma-mm	ITP	TI
A	12.47	6.24	10.18	5.09	11.33	70.17	22.92	33.27
B	11.98	5.99	8.12	4.06	10.05	69.06	27.21	29.10
C	16.27	8.14	20.33	10.16	18.30	41.50	3.31	88.20
D	7.56	3.78	6.47	3.24	7.02	76.39	50.36	18.20
E	2.04	1.02	1.79	0.89	1.92	100.16	5536.02	3.83
F	2.07	1.04	1.17	0.58	1.62	120.10	6151.10	3.09
G	5.17	2.59	-	-	2.59	20.09	-	-
H	4.16	2.08	2.42	1.21	3.29	86.07	253.90	7.65
I	3.94	1.97	2.39	1.19	3.17	80.15	153.03	7.89
J	22.44	11.22	28.59	14.29	25.51	44.44	2.79	114.87
K	8.41	4.21	6.87	3.44	7.80	58.51	21.04	26.11
L	10.60	5.30	7.19	3.59	8.89	72.42	35.93	24.56
M	13.60	6.66	25.87	12.93	19.59	43.64	3.29	89.84
N	4.13	2.06	2.00	1.00	3.07	100.20	4974.80	6.12
O	22.38	11.40	36.49	18.24	29.64	40.41	1.84	145.68
P	15.04	7.52	-	-	7.52	30.20	-	-
Q	3.64	1.82	2.08	1.04	2.86	88.60	317.13	6.53

Table 20: Pearson correlation coefficient (r-values) in *Clarias gariepinus*

Treat-ments	r-mm vs k-mm	r-mm vs Ma-mm	r-mm vs ITP	k-mm vs Ma-mm	Ma-mm vs ITP	k-mm vs ITP
A	-	0.64	-	-	-	-0.70
B	-	-	-	0.68	-	-0.93
C	0.67	-	-	-	-	-
D	-	-	-	-	-	-0.85
E	-	-	-	-	-	-0.99
F	-	-	-	-	-	-0.99
G	-	-	-	-	-	-
H	-	-	-	-	-	-1.00
I	-	-	-	-	-	-0.99
J	-	-	-	-	-	-
K	-	-	-	-	-	-0.99
L	-	-	-	-	-	-0.80
M	-	-	-	-	-	-0.97
N	-	-0.55	-	-	-	-0.98
O	-	-	-	-	-0.54	0.53
P	-	-	-	-	-	-
Q	-	-	-	-	-	-0.98
	1	3	0	1	1	14

Table 21 : Pearson correlation coefficient (r-values) in *Schilbe (s) mystus depressirostris*

Treat-ments	r-mm vs k-mm	r-mm vs Ma-mm	r-mm vs ITP	k-mm vs Ma-mm	Ma-mm vs ITP	k-mm vs ITP
A	-	0.67	-	-	-	-0.91
B	-	-	-	0.59	-	-0.94
C	0.59	-	-	-	-	-0.90
D	-	-	-	-	-	-0.99
E	-	-	-	-	-	-0.99
F	-	-	-	-	-	-0.94
G	-	-	-	-	-	-
H	-	-	-	-	0.58	-0.99
I	-0.57	-	-0.56	-	-	-0.99
J	-	-	-	-	-	-0.99
K	-	-	-	-	-	-0.97
L	-	-	-	-	-	-0.76
M	-	-	-	-	-	-0.86
N	-0.63	0.64	0.65	-	-	-
O	-	-	-0.65	-	0.62	-0.98
P	-	-	-	-	-	-0.76
Q	-	-	-	-	-	-0.99
	3	3	4	1	2	15

Table 22: Pearson correlation coefficient for TEG parameters recorded for *Schilbe (S) mystus depressirostris.*

Parameter	k-mm	r-mm	Ma-mm	ITP
k-mm	-	0.89	-0.68	-0.54
r-mm	0.89	-	-0.81	-
Ma-mm	-0.67	-0.81	-	0.72
ITP	-0.54	-	0.72	-

Table 23: Pearson correlation coefficients for TEG parameters recorded for *Clarias gariepinus*

Parameter	k-mm	r-mm	Ma-mm	ITP
k-mm	-	0.93	-0.69	-0.54
r-mm	0.93	-	-0.85	-
Ma-mm	-0.69	-0.85	-	0.72
ITP	-0.54	-	0.72	-

Table 24: The effects of stress on Lee-White Clotting times *in Clarias gariepinus* and *Schilbe (S) mystus depressirostris*

	Clarias gariepinus				*S. (s) mystus depress.*			
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**
Normal	141.29	0.82	140.20	143.30	128.57	0.56	127.40	129.40
Chronic	62.78	1.60	60.20	66.40	53.52	1.31	51.40	56.20
Acute	24.18	0.58	23.40	25.20	11.03	0.78	10.10	12.80

The effects of various Thromboplastins on Prothrombin times in *Clarias gariepinus* and *Schilbe (S) mystus depressirostris*

Table 25: *Clarias gariepinus* thromboplastin (n = 10)

	Clarias gariepinus				*S. (S) mystus depress.*			
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**
Normal	38.09	0.59	37.20	38.90	34.67	0.86	33.40	35.60
Chronic	25.46	0.10	25.30	25.60	21.39	2.02	20.20	26.80
Acute	23.86	1.18	23.00	26.50	18.87	0.44	18.10	19.40

Table 26: *Schilbe (S) mystus depressirostris* thromboplastin

	Clarias gariepinus				*S (S) mystus depress.*			
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**
Normal	33.51	1.58	30.40	35.40	18.46	0.15	18.20	18.70
Chronic	22.57	0.17	22.40	22.80	15.57	0.15	15.40	15.80
Acute	20.49	0.74	19.80	22.30	15.18	0.35	14.50	15.60

Table 27: Rabbit Brain Thromboplastin

	Clarias gariepinus				*S (S) mystus depress.*			
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**
Normal	128.84	2.21	125.60	134.50	98.41	0.11	98.20	98.60
Chronic	85.97	2.89	80.40	89.40	38.14	1.56	35.40	40.80
Acute	39.96	1.72	35.20	41.60	35.45	1.02	33.60	37.40

Table 28: Mean values for clotting factors in the two freshwater fish species.

	Clarias gariepinus			*S. (S) mystus depress*		
	Normal	**Chronic**	**Acute**	**Normal**	**Chronic**	**Acute**
Factors	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**
II	53.10	38.53	36.78	25.43	15.78	13.47
V	48.92	44.18	40.36	44.62	42.24	40.57
VII	47.97	45.92	42.85	46.41	22.80	21.98
X	28.61	28.59	23.29	34.27	31.21	28.41
VIII	44.70	40.48	37.44	34.59	29.49	23.24
IX	67.53	54.64	50.99	48.89	39.19	23.01
XI	66.46	53.16	49.42	44.46	42.12	42.86
XII	58.31	54.21	49.26	34.79	29.68	27.53

FIGURES

FIG.1 A schematic representation of Intrinsic and Extrinsic pathways for Blood Coagulation.

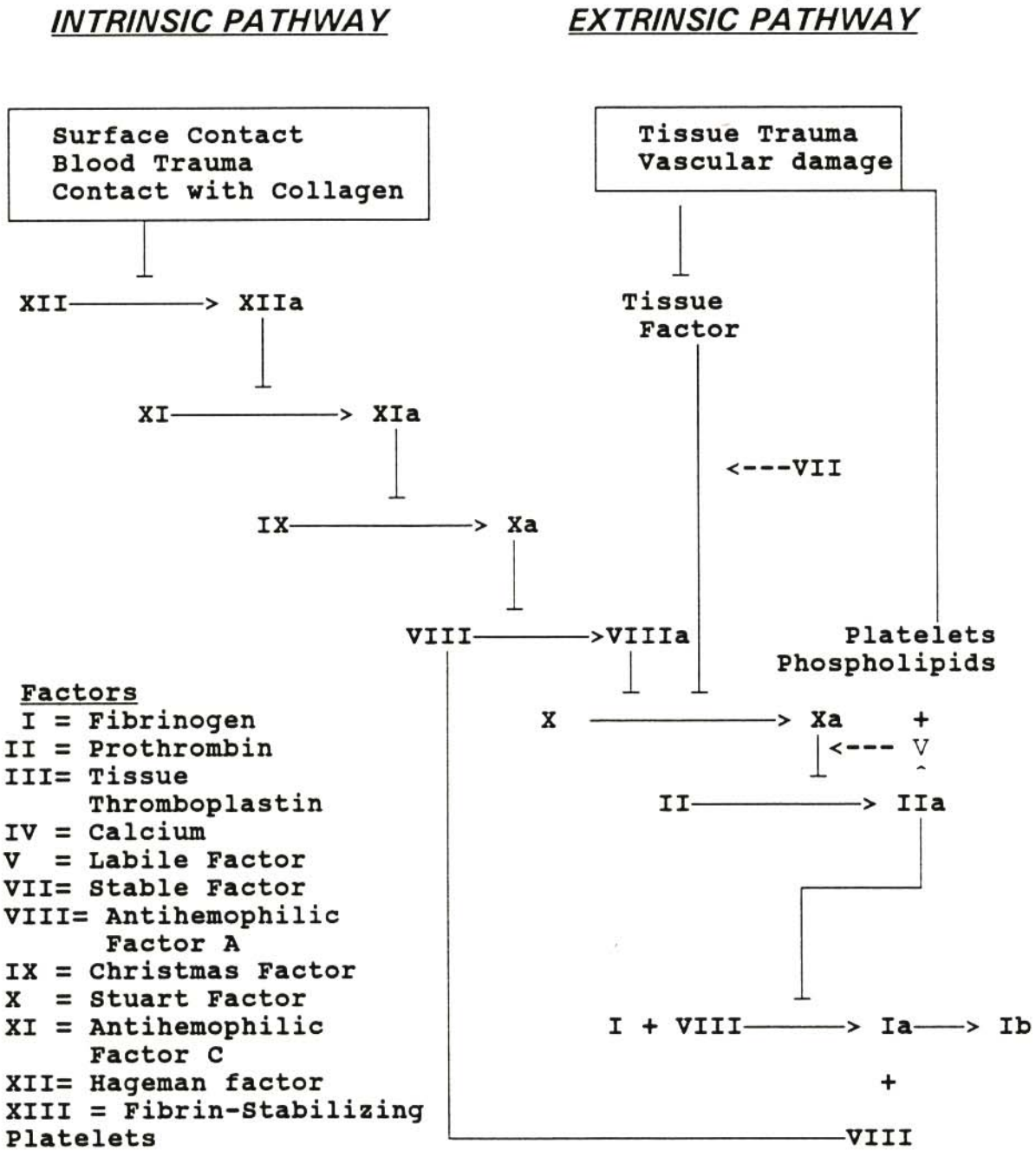
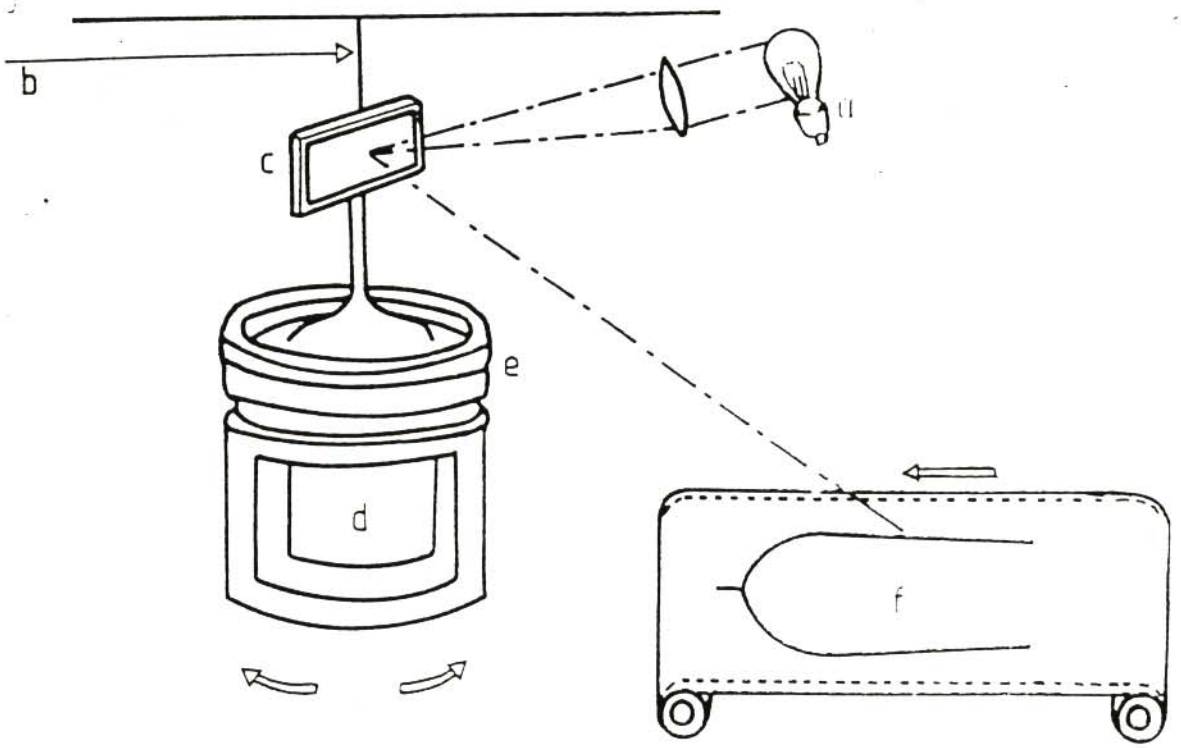


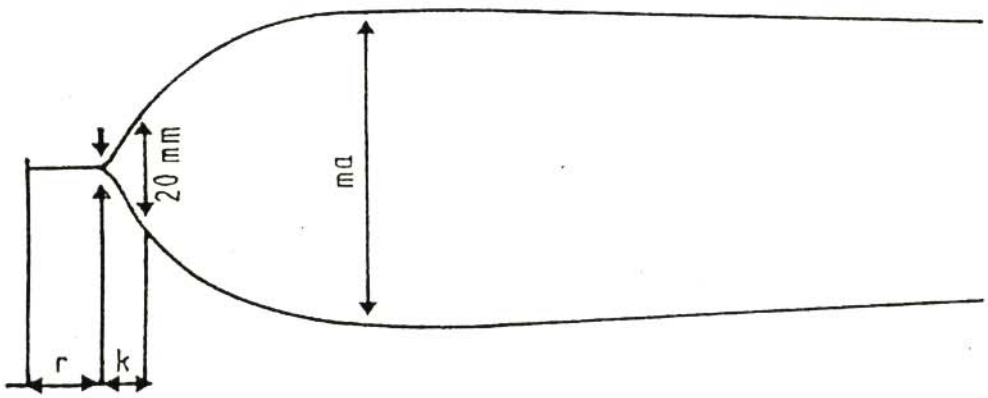
FIG. 2 Principles of operations of the Thrombelastography



a. Light source b. Torsion wire c. Mirror

d. Piston e. Cuvette f. Thromboelastogram

FIG. 3 Normal Thrombelastogram of human blood.



BLOOD COAGULATION AND TEG RECORDINGS

Fig. 4. Effects of Neutralized MS 222

Fig. 5. Effects of Electronarcosis

Fig. 6. Effects of Natural MS 222

Fig. 7 Effects of Natural MS 222 added to blood

Fig. 8. Effects of Neutralized MS 222 without TSC

Fig. 9. Effects of Natural MS 222 without TSC

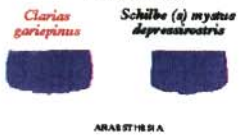


Fig. 10. Effects of Stress without TSC added

Fig. 11. Effects of Acute Stress

Fig. 12. Effects of Chronic Stress

Fig. 13. Effects of Cortisol

Fig. 14. Effects of Adrenalin

Fig. 15. Effects of Noradrenalin

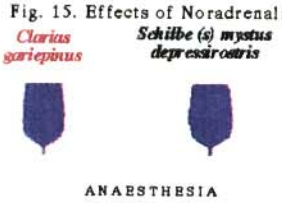


Fig. 16. Effects of Adrenaline and Cortisol

Fig. 17. Effects of Cortisol and Noradrenaline

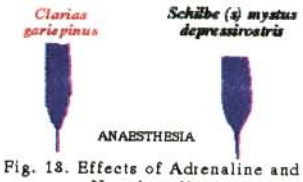


Fig. 18. Effects of Adrenaline and Noradrenaline

Fig. 19. Effects of Adrenaline, NA and Cortisol

Fig. 18. Effects of Adrenaline and Noradrenaline

Fig. 20. Effects of A, Cortisol and 1/2NA

Fig. 21: Whole blood thrombocyte aggregatory behaviour in *Clarias gariepinus*

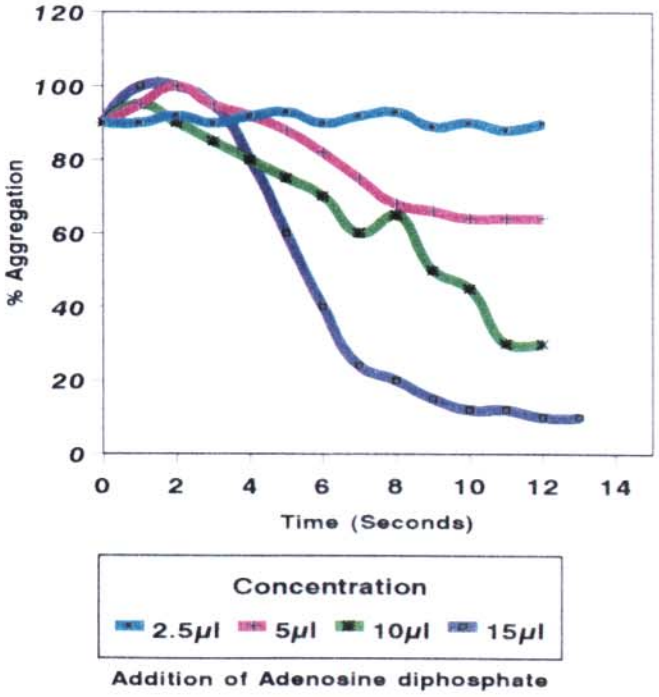


Fig. 22: Whole blood thrombocyte aggregatory behaviour in *Schilbe (S) mystus depressirostris*

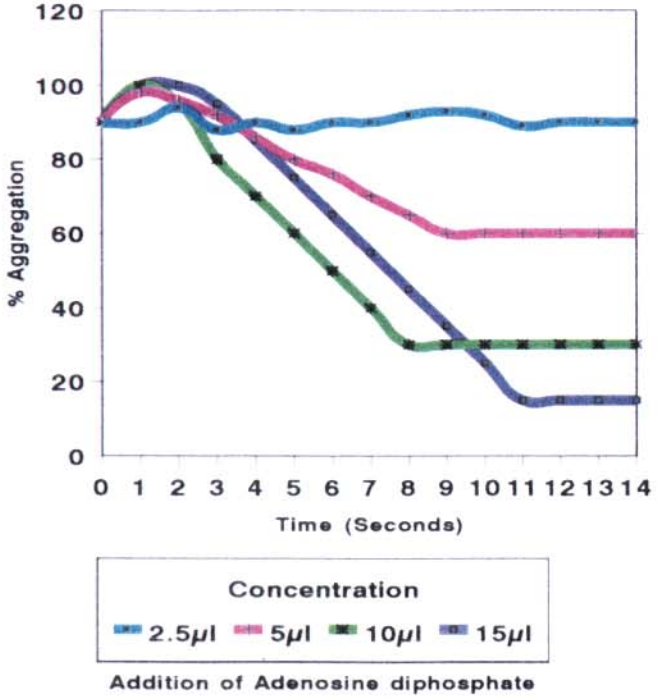


Fig. 23: Whole blood thrombocyte aggregatory behaviour in *Clarias gariepinus*

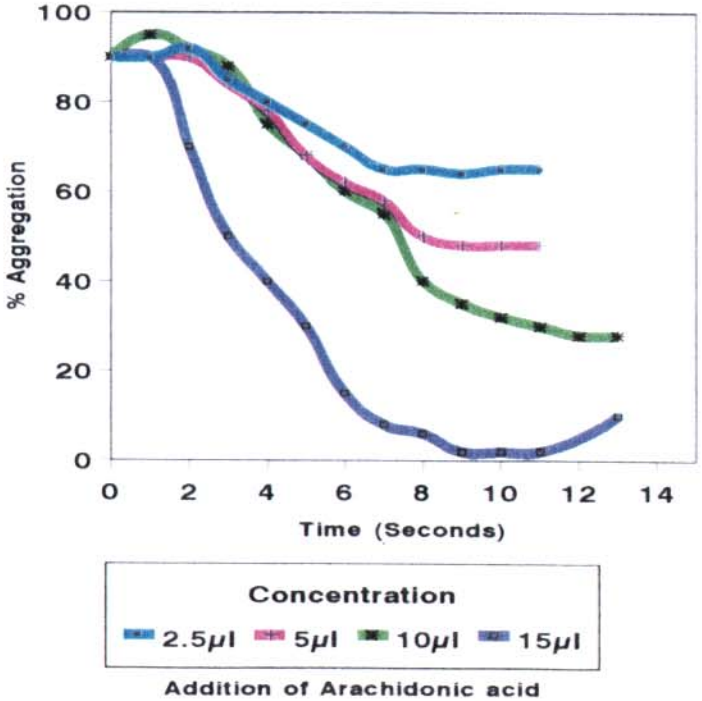


Fig. 24: Whole blood thrombocyte aggregatory behaviour in *Schilbe (S) mystus depressirostris*

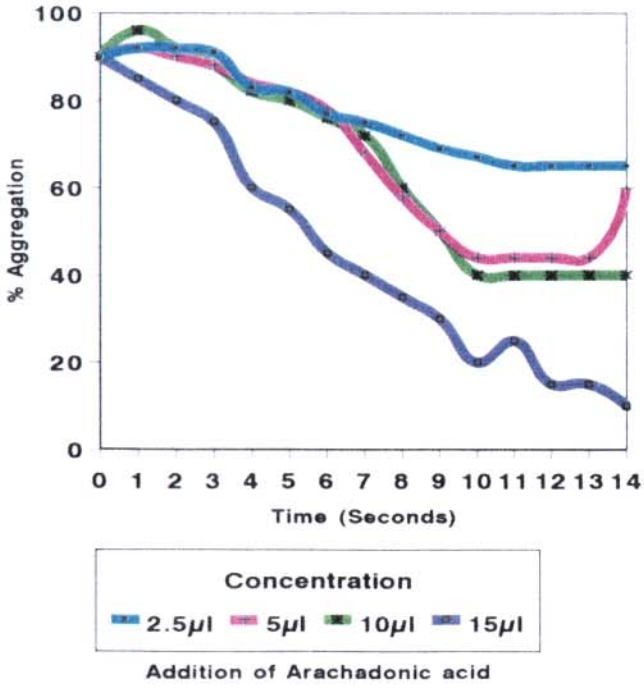


Fig. 25: Whole blood thrombocyte aggregatory behaviour in *Clarias gariepinus*

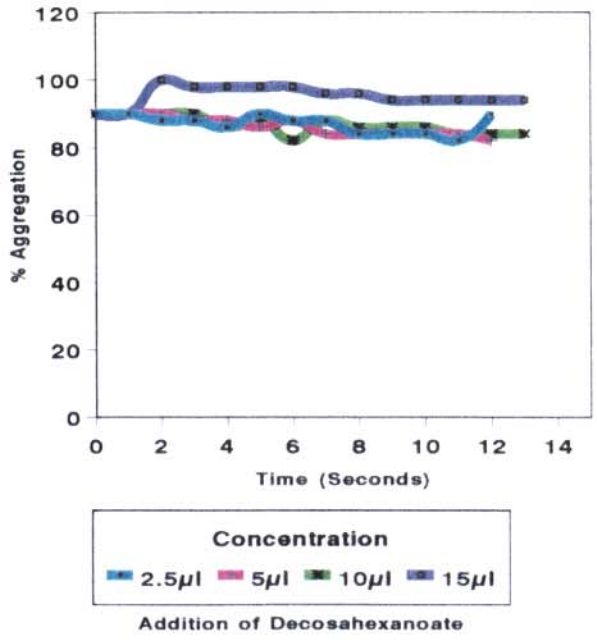


Fig.26: Whole blood thrombocyte aggregatory behaviour in *Schilbe (S) mystus depressirostris*

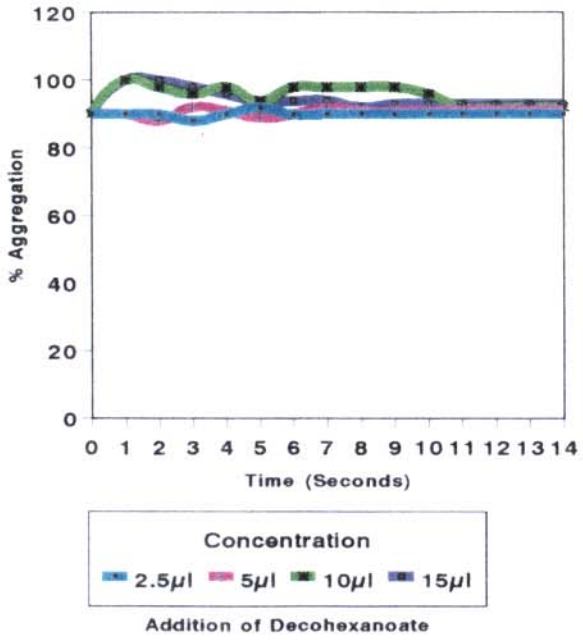


Fig. 27 Whole blood thrombocyte aggregatory behaviour in *Clarias gariepinus.*

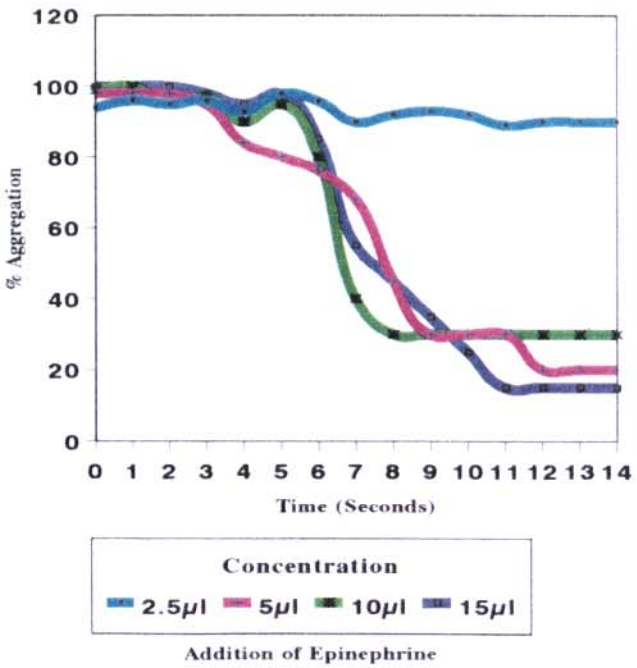
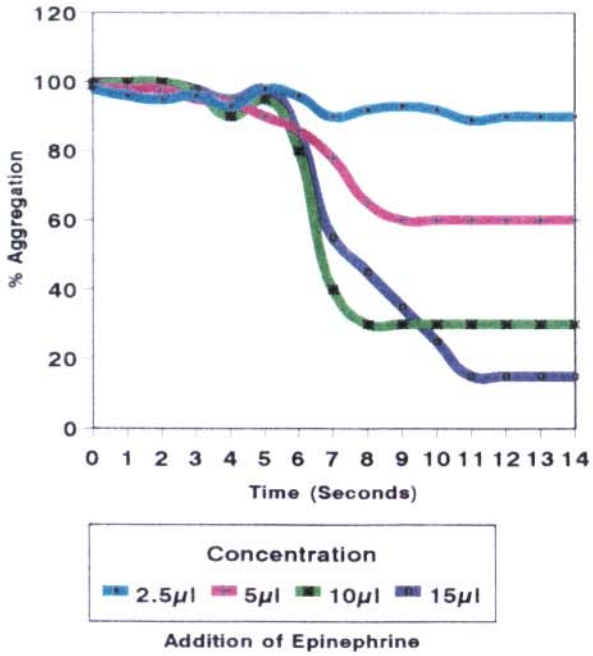


Fig. 28: Whole blood thrombocyte aggregatory behaviour in *Schilbe (S) mystus depressirostris*



TABLES AND FIGURES

Fig. 29 Clotting factor times for *Clarias gariepinus*

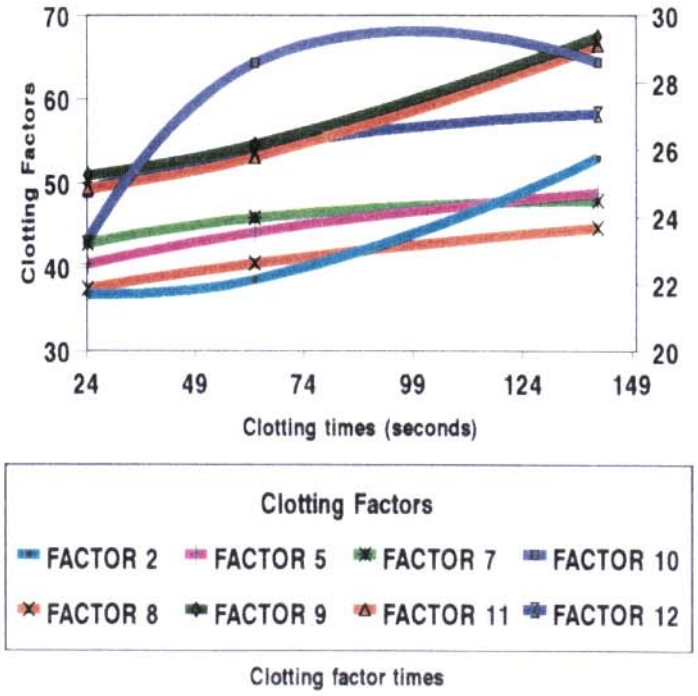


Fig. 30: Clotting factor times for *Schilbe (S) mystus depressirostris*

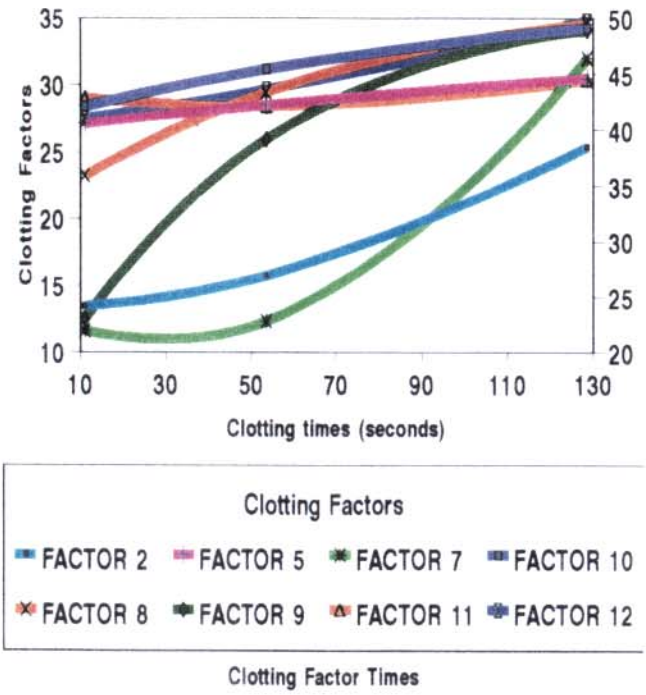


Fig. 31: Linear relationships for TEG parameters in *Clarias gariepinus*

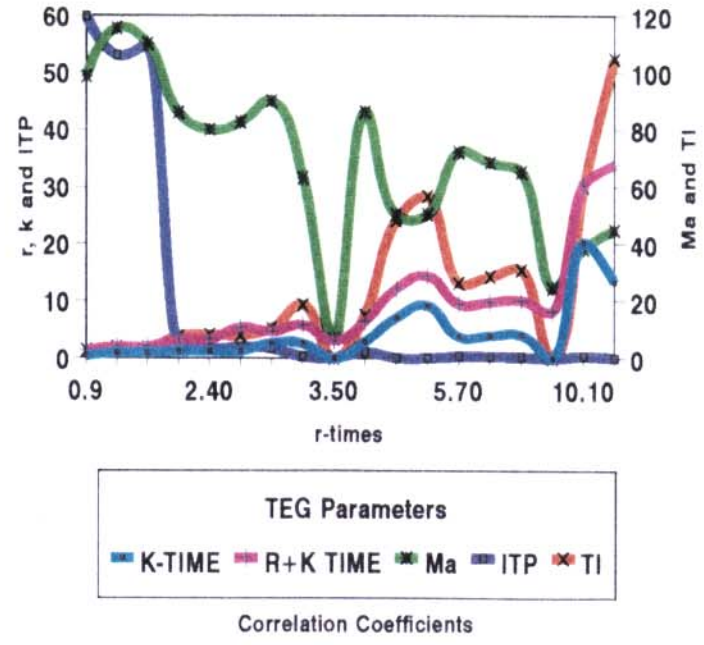
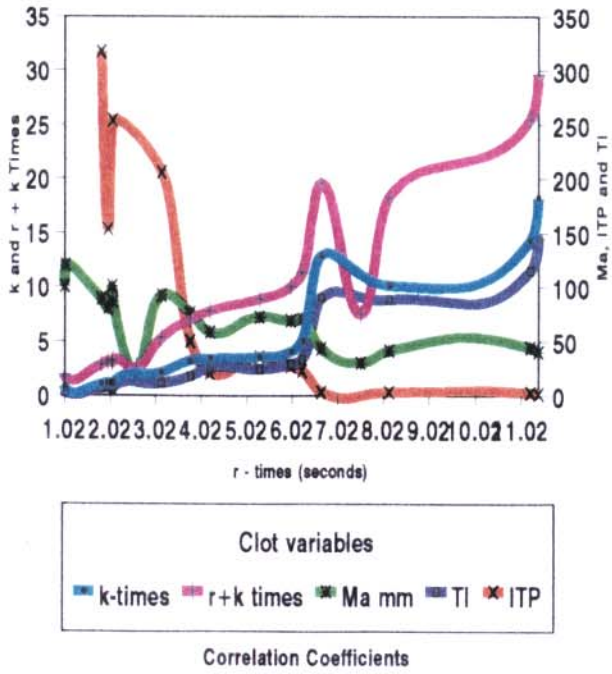


Fig. 32: Clot variable relationships for *Schilbe (S) mystus depressirostris*



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