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Evaluation of the Oxidative Stress in Fish Oocyte

Bachelor thesis

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Annotation

This work aims to identify the role of oxidative stress in the progress of oocyte ageing in African catfish *Clarias gariepinus* by measuring the levels of thiobarbituric acid reactive substances (TBARS) and carbonyls as indicators of lipid oxidation and protein oxidation respectively using spectrophotometric assays.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 10.12.2021.

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Tamás Beretka

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Abstract

Post-ovulatory oocyte ageing which is defined as the time interval between ovulation and fertilization has been identified as the most important factor affecting egg quality after ovulation in several fish species. The molecular mechanisms of oocyte aging are not well defined. In other vertebrates, oxidative stress as a consequence of an increase in reactive oxygen species (ROS) has been proposed as the initiating factor of oocyte ageing which triggers a cascade of events finally leading to loss of fertility. To identify the role of oxidative stress in the progress of oocyte ageing in African catfish *Clarias gariepinus* the levels of thiobarbituric acid reactive substances (TBARS) and carbonyls as indicators of lipid oxidation and protein oxidation respectively were measured using spectrophotometric assays. Stripped ova from females were stored separately at 25 °C. The stored ova were fertilized at 0, 2, 4, 8 and 16 hours post-stripping (HPS) and the egg viability percentages and larval malformation rates were examined. A complete loss of egg viability occurred at 16 hours post-stripping (HPS). The embryo mortality and larval malformation rates increased significantly over time and were the highest in the most aged oocytes. Postovulatory ageing of African catfish oocytes for up to 16 hours after oocyte ovulation exhibited no effect on lipid oxidation. Comparison of the protein oxidation status between freshly ovulated oocytes and aged oocytes revealed constant values for carbonyls marker. These results indicate no involvement of oxidative stress during African catfish oocyte ageing at least until the complete loss of egg fertilizing ability. Additional tests and analysis, however, are needed to entirely investigate the role of oxidative stress in defects of egg quality during oocyte ageing.

Keywords: *Clairas gariepinus*, egg viability, oocyte ageing, oxidative stress

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Abbreviations

ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
HPS	Hours post-stripping
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MII	Metaphase II
MPF	Maturation (or M-phase) promoting factor
mRNA	Messenger ribonucleic acid
OS	Oxidative stress
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances

1. Introduction

1.1. Fish Oocytes

A female gamete or unfertilized egg is an oocyte released from the ovary after the second meiotic division (Bobe & Labbé, 2010), it is the final product of the oogenetic process that occurs within the ovary through oogenesis (Tata, 1986). Oogenesis occurs in the ovarian follicle where the primary ovarian follicle differentiates into an egg and acquires the capability of forming a viable embryo after fertilization. Massive structural and functional changes accompany this process (Lubzens et al., 2010), the incorporation, synthesis, and processing of egg components that occur during oogenesis play a key role in the coordinated assembly of a good quality oocyte that will, once fertilized, develop into a normal embryo (Bobe & Labbé, 2010).

In primitive fishes, matured eggs are released into the abdominal cavity, while in most bony fish eggs are ovulated into the ovarian lumen, and they remain there until spawning is stimulated by environmental factors or eggs are collected by artificial techniques. In the period after ovulation, eggs are bathed in a fluid known as the ovarian fluid (Samarin et al., 2015).

In vertebrates, the metaphase II oocyte released from the ovary contains yolk proteins, vitamins and hormones (Bobe & Labbé, 2010) as well as DNA and RNA polymerases, histone and non-histone chromatin proteins, transcription and translation factors, rRNAs, tRNAs, and maternally inherited mRNAs (Tata, 1986). After fertilization, those maternal factors support early embryonic development until activation of zygotic transcription and thus have a crucial role during early embryogenesis (Bobe & Labbé, 2010). In fish, as in other lower vertebrates, zygotic transcription is initiated during the mid-blastula stage and is also known as the ‘‘mid-blastula transition” (MBT). Differential degradation of the maternally inherited mRNAs takes place in parallel to the activation of zygotic transcription (Lubzens et al., 2010). Therefore, the nature and abundance of maternal mRNAs stored in the eggs appears to be important for the functionality and competence of the oocyte (Bobe & Labbé, 2010).

1.2. Fish Oocyte Quality

In aquaculture and other animal breeding programs, controlling the quality of eggs is of significant interest and relevance (Samarin et al., 2019a). Fish egg quality is defined as the ability of the egg to be fertilized and subsequently develop into a normal embryo (Bobe & Labbé, 2010). Oocyte integrity and health is of great importance for embryo quality and the later life of the offspring as the oocyte contains factors responsible for orchestrating embryogenesis and remodeling the parental genome (Samarin et al., 2019a). The oocyte is also the only source of mitochondria in the developing embryo (Lord et al., 2013).

The quality of fish gametes in the wild or in captivity is highly variable (Aegerte & Jalabert, 2004). It is dependent on several intrinsic and environmental factors. As a result, the topic of gamete quality has gotten a lot of attention. Gamete quality can be estimated using biologically and biotechnologically relevant parameters (Bobe & Labbé, 2008).

1.2.1. Quality Estimation

Apart from markers of extremely low quality, no effective marker or estimator of oocyte quality prior to fertilization exists. Non-viable oocytes can sometimes be identified in some species through the assessment of morphological changes but significant differences in egg quality are also observed that cannot be linked to simple morphological criteria. Thus, monitoring embryonic and larval survival after fertilization is the only biologically relevant way to accurately estimate oocyte quality (Bobe & Labbé, 2010).

Depending on fish species fertilization rate can be hard to monitor. Fertilization success is not necessarily reflective of further developmental success; hence it is important to record survival through development as well as monitor embryonic and larval deformities at specific stages to completely assess gamete quality (Bobe & Labbé, 2010).

1.2.2. Factors Affecting Oocyte Quality

Although the environmental control of gamete quality is far from being fully understood, a number of external, experimental and rearing factors that influence gamete quality have been identified (Bobe & Labbé, 2010).

Even though it is well accepted that broodstock nutrition can significantly impact reproductive performance data on the specific effects of broodstock diet on gamete quality are more limited (Brooks et al., 1997). Environmental factors such as temperature, salinity and photoperiod have been shown to have an impact on reproductive success. It is, however, difficult to separate specific effects of temperature and photoperiod on egg quality (Bobe & Labbé, 2010). High temperature, in addition to the negative impact on egg quality by itself, also further decreased egg quality resulting from the post-ovulatory ageing process (Aegerter & Jalabert, 2004). Exposure to xenobiotics and pollutants, or physiochemical properties of the water can affect gamete quality (Brooks et al., 1997).

In aquaculture, hormonal induction of spawning is widely used to induce ovulation in species that do not spontaneously ovulate in captivity, or to synchronize ovulation for practical reasons in other species. The later development of ovulated oocytes greatly depends on the ovarian stage at the time of spawning induction. For the purpose of artificial fertilization, eggs can be collected after ovulation from the females by stripping. In the time period between ovulation and fertilization, post-ovulatory ageing occurs in the oocyte (Bobe & Labbé, 2010).

During post-ovulatory ageing morphological, physiological, biochemical, histological, cellular, and molecular changes occur inside the egg (Samarin et al., 2015). Oocyte ageing after ovulation has been identified as the most important factor affecting egg quality in several fish species (Rime et al., 2004).

1.3. Post-ovulatory Oocyte Ageing

Between the release of the metaphase II (MII) oocyte from the follicle at ovulation and fertilization post-ovulatory ageing occurs (Bobe & Labbé, 2010). Post-ovulatory ageing, also referred to as ‘‘over-ripening”, is a process distinct from “ovarian ageing” which occurs within the ovary of females towards the end of reproductive life, as they approach the climacteric (Lord & Aitken, 2013). Fish display a vast diversity of reproductive modes, and compared to other animals, most species produce a high number of oocytes which makes them good model animal to evaluate oocyte ageing (Samarin et al., 2019a).

The MII oocytes can be retained inside the fish body (*in vivo* storage of eggs) or kept outside it (*in vitro* storage of eggs) (Samarin et al., 2016). After ovulation and/or stripping an optimal time window for fertilization exists. Depending on the fish species and water temperature, the optimal time, may vary from a few minutes to a few weeks (Samarin et al., 2019a). Post-ovulatory ageing has been associated with an overall decrease in the oocyte’s ability to be fertilized and to subsequently develop into a normal embryo (Lord & Aitken, 2013). The decrease in developmental capacities can happen without any noticeable morphological changes in the appearance of the egg. Depending on the fish species and external factors, egg quality decreases more or less rapidly. However, the drop in egg quality occurs very rapidly after ovulation in most fish species (Bobe & Labbé, 2010).

Morphological, physiological, biochemical and histological consequences of post-ovulatory oocyte ageing have largely been established, but the molecular mechanisms controlling this process are not well defined (Samarin et al., 2019a). Cumulus cell, critical cell cycle factors MPF and MAPK and mitochondrial dysfunction are known to influence the onset of post-ovulatory ageing (Lord & Aitken, 2013).

1.3.1. MPF and MAPK

Maturation (or M-phase) promoting factor (MPF) and mitogen-activated protein kinase (MAPK) are kinases that control the resumption of meiotic division from the prophase I arrest and almost all of the morphological changes that occur during oocyte maturation (Kotani & Yamashita, 2002). Their activation is essential for the maintenance of metaphase II arrest (Fan & Sun, 2004).

During post-ovulatory ageing of MII oocytes, a gradual decline in the concentration of MPF and MAPK has been observed and has been associated with increased levels of parthenogenetic activation and fragmentation (Lord & Aitken, 2013).

The mechanism of MPF degradation during post-ovulatory ageing has been studied extensively. MPF is comprised of two molecules: the catalytic subunit $p34^{cdc2}$ and the regulatory subunit cyclin B. When MPF is in its active form, the two subunits are coupled and T-161 of the catalytic subunit is phosphorylated. MPF is inactivated after fertilization by the dephosphorylation of T-161 and degradation of the decoupled cyclin B (Lord & Aitken, 2013). In oocytes undergoing post-ovulatory ageing the inactivation of MPF takes place via an alternate pathway. Inactivation of MPF is mediated by the phosphorylation of T-14 and/or T-15 of $p34^{cdc2}$ which leads to the accumulation of the inactive compound "pre-MPF," in which the two subunits are still bound (Kikuchi et al., 2002).

When nonaged oocytes were incubated with vanadate, an inhibitor of tyrosine phosphatase, accelerated formation of pre-MPF and increased susceptibility to parthenogenetic activation and fragmentation were observed, while treatment of oocytes with caffeine, reduced $p34^{cdc2}$ phosphorylation and increased active MPF levels leading to decreased parthenogenetic activation and fragmentation (Kikuchi et al., 2002) and extended the window of fertilization (Ono et al., 2011).

1.3.2. Cumulus Cells

Cumulus cells surround the oocytes during maturation, ovulation, and fertilization. Their function has been extensively studied, but little is known about their role in oocyte ageing. During *in vitro* ageing, MII oocytes enclosed within the cumulus cell complex experienced an increase in spontaneous activation and fragmentation, accelerated decline of MPF (Miao et al., 2005) and decreased levels of blastocyst formation (Wu et al., 2011) when compared to oocytes that have been denuded of their cumulus cells.

Several explanations for cumulus cell-associated acceleration of post-ovulatory ageing have been proposed (Lord & Aitken, 2013). It has been hypothesized that soluble paracrine factor(s) that promote post-ovulatory ageing are secreted by cumulus cells, an event that potentially coincides with the entry of the cumulus cells into apoptosis (Wu et al., 2011). In addition, oocyte metabolism, and thus post-ovulatory ageing, appear to be affected by the accelerated depletion of the oocyte metabolite pyruvate from culture medium by cumulus cells (Lord & Aitken, 2013).

An alternative explanation is that mitochondrial dysfunction and subsequent ageing and apoptosis in post-ovulatory oocytes could be caused by the bioactive sphingolipid, ceramide which is generated by the cumulus cells (Perez et al., 2005).

Cumulus cells clearly exacerbate degeneration in ageing post-ovulatory oocytes. However, since oocytes that have been denuded of their cumulus cells also experience ageing and apoptosis additional mechanisms must be at play in controlling this process (Miao et al., 2005, Lord et al., 2013).

1.3.3. Mitochondrial Dysfunction

Mitochondrial dysfunction is another factor involved in post-ovulatory ageing of oocytes (Lord & Aitken, 2013). Mitochondria are the primary source of adenosine triphosphate (ATP) production within oocytes as well as early embryos and are thus crucial for normal oocyte function (Dumollard et al., 2007). However, during post-ovulatory ageing the functionality of the mitochondria becomes compromised. This was demonstrated in *in vitro* aged oocytes where, a loss of mitochondrial membrane potential (Zhang et al., 2011) and a decline in levels of ATP production (Chi et al., 1988) have been reported.

The influence of mitochondria on oocyte fate was tested by microinjecting isolated mitochondria into oocytes. After culturing for 24 hours, MII oocytes whose total mitochondrial pool was increased by 5% via microinjections showed a significant decline in levels of apoptosis when compared with control oocytes. This demonstrates the strong control that mitochondrial function has over post-ovulatory ageing (Perez et al., 2000).

Mitochondrial dysfunction is likely the link between post ovulatory ageing and apoptosis. Damage to the mitochondria is known to cause an increased production of reactive oxygen species (ROS) (Lord & Aitken, 2013) and the release of pro-apoptotic factors such as cytochrome c (Liu et al., 2009a).

1.4. Reactive Oxygen Species and Oxidative Stress

It is currently unclear whether an initiating factor triggers a cascade of events finally leading to loss of fertility, or if several different biochemical and functional changes occur separately to create an “aged” MII oocyte (Lord & Aitken, 2013). Studies of higher vertebrates suggest that oxidative stress caused by the increase in reactive oxygen species (ROS) may act as the initiator for a series of events that result in the “aged oocyte” phenotype (e.g., Tarín et al., 2000; Takahashi et al., 2003; Lord et al., 2013).

1.4.1. ROS

Reactive oxygen species (ROS) comprise a broad category of both free radical and non-radical reactive oxygen derivatives (Li et al., 2016). A free radical can be defined as an

atom or molecule capable of independent existence that contains one or more unpaired electrons. Free radical species are unstable and highly reactive. They become stable by acquiring electrons from any nearby molecule. This leads to the attacked molecule losing its electron and becoming a free radical itself, beginning a cascade of chain reactions resulting in widespread injury to biomolecules, cellular damage and disease (Phaniendra et al., 2015). Similar to ROS, the term reactive nitrogen species (RNS) has been coined to include nitrogen-containing reactive species. RNS are almost exclusively oxygen-containing species and can thus be considered as a subclass of ROS (Agarwal & Prabakaran, 2005).

In oocytes, as in other cells, ROS are important mediators of intracellular signaling responsible for numerous cellular functions under physiological conditions (Nasr-Esfahani & Johnson, 1991).

ROS can originate from either endogenous or exogenous sources. Intracellular sources of ROS include different cellular organs where oxygen consumption is high such as mitochondria, peroxisomes and endoplasmic reticulum as well as nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases. However, most of the intracellular ROS production occurs in the mitochondria as a by-product of oxidative phosphorylation (Phaniendra et al., 2015)

ROS can also be overproduced in the oocyte microenvironment in response to several conditions, such as ongoing acute or chronic infections or inflammation, certain medications, radiation and pollutants (Goud et al., 2008).

The accumulation of ROS, in particular the hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\bullet-}$) and peroxynitrite ($OONO^-$), in oocytes over time following ovulation has been shown both *in vivo* and *in vitro* (Takahashi et al., 2003, Lord et al., 2013).

Superoxide anion radical is the most important widespread ROS. It can exist in two forms; as $O_2^{\bullet-}$ or as a hydroperoxyl radical (HO_2) at low pH. The hydroperoxyl radical is the most important form as it can enter the phospholipid bilayer more easily than the charged form ($O_2^{\bullet-}$). Most of the intracellular superoxide radical production occurs at two major sites in the mitochondria; complex I (NADH dehydrogenase) and complex III (ubi-quinone cytochrome c reductase) (Figure 1). As a result of the transfer of electrons from complex I or II to coenzyme Q or ubiquinone (Q) a reduced form of coenzyme Q is formed (QH_2). In the Q-cycle coenzyme Q is regenerated from QH_2 via an unstable intermediate semiquinone anion ($Q^{\bullet-}$). Electrons are transferred from $Q^{\bullet-}$ to molecular oxygen leading to the formation of superoxide radical. Since the generation of superoxide is non-enzymatic that means that higher the metabolic rate, the greater is the production of the ROS (Finkel & Holbrook, 2000).

Mitochondrial superoxide dismutase (MnSOD) converts the superoxide anion to hydrogen peroxide. In this reaction, one radical is oxidized to oxygen and the other is reduced to hydrogen peroxide. Catalase (CAT) and glutathione peroxidase (GPx) can detoxify H_2O_2 (Phaniendra et al., 2015).

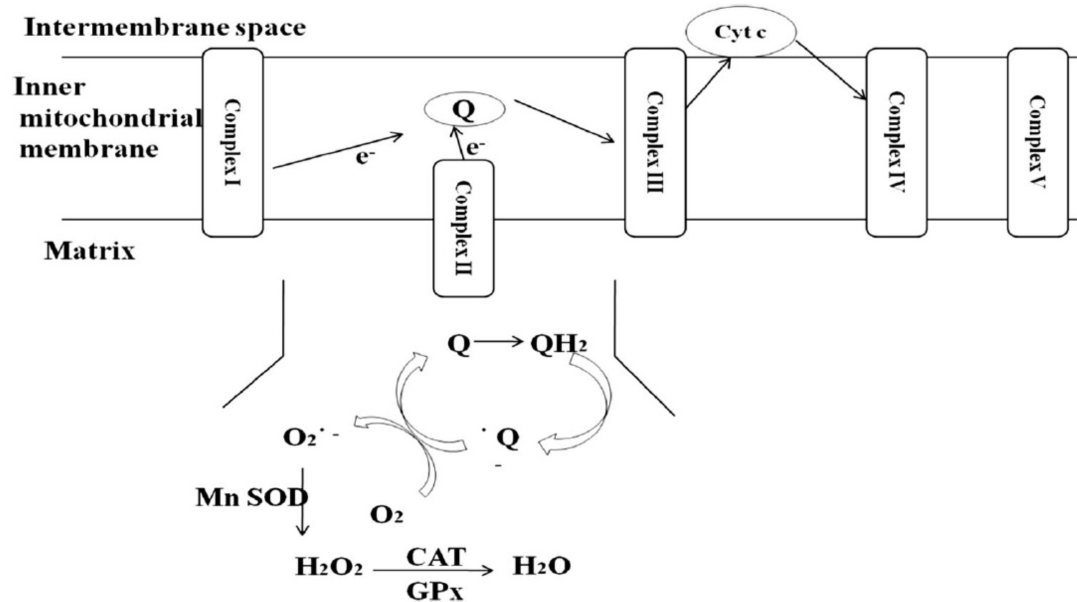

Figure 1 Mitochondrial ROS production (Source: Phaniendra et al., 2015)

Hydrogen peroxide is not a free radical, but it can cause damage to the cell at relatively low concentration (10 µM). It is physiologically important among ROS due to the fact that its lifetime in the intracellular space is relatively long and it is freely diffusible through biological membranes. Even though H_2O_2 has no direct effect on DNA, it can damage DNA by producing the more toxic hydroxyl radical in the presence of transition metal ions (Phaniendra et al., 2015).

The levels of ROS in a biological system are determined not only by the rates of their production, but also by the presence and activities of cellular antioxidant defenses (Li et al., 2016). An imbalance between the production of ROS and the inherent antioxidant defense leads to oxidative stress (Phaniendra et al., 2015).

1.4.2. Oxidative Stress

Oxidative stress, the term initially coined by Helmut Sies in 1985, refers to a condition where the levels of ROS significantly overwhelm the capacity of antioxidant defenses, leading to potential damage in a biological system. In a biological system, oxidative stress can be caused by either increased ROS formation or decreased activity of antioxidants or both (Li et al., 2016).

While the oocyte does offer some intracellular protection against ROS in the form of the antioxidant glutathione (GSH), the intracytoplasmic level of GSH decreases in aged oocytes with post-ovulatory age (Boerjan & de Boer, 1990). When it comes to the increase in ROS production, it has been theorized that, it occurs due to the effects of hypoxia on the mitochondria electron transport chain, although the precise mechanism is still a matter of debate (Tafani et al., 2016).

1.5. Pathways of Post-ovulatory Ageing

As a consequence of the progressive increase in ROS production and the associated depletion of antioxidant protection, the post-ovulatory aged oocyte experiences a state of oxidative stress. (Lord & Aitken, 2013). The hypothesis that oxidative stress could be the initiator of post-ovulatory ageing in MII oocytes is supported by the finding that the onset of oxidative stress precedes the appearance of markers of ageing and apoptosis in oocytes *in vitro*. Oxidative stress may occur in mouse oocytes after as little as 8 h in culture (Lord et al., 2013).

Oxidative stress could initiate the post-ovulatory ageing process via multiple pathways (Figure 1): ROS-induced mitochondrial dysfunction, impaired calcium homoeostasis, a decline in the levels of critical cell cycle factors such as the maturation-promoting factor, lipid oxidation and global damage to DNA and proteins have all been linked to oxidative stress (Lord & Aitken, 2013). Thus, reduced fertilization success, diminished embryo quality and increased abnormalities observed in the later life of the offspring have been associated with increased oxidative stress in oocytes (Tarín et al., 2000, Lord & Aitken, 2013).

Additionally, oxidative stress can directly influence the onset of apoptosis in post-ovulatory aged oocytes (Lord et al., 2013). The *in vitro* treatment of MII oocytes with H_2O_2 has been shown to cause a decline in levels of the anti-apoptotic molecule Bcl-2 (Takahashi et al., 2009) induce expression of pro-apoptotic Bax and caspase-3 (Chaube et al., 2005). as well as cytochrome c release (Liu et al., 2000). These pathologies are common to both oxidative stress and oocyte ageing (Lord et al., 2013).

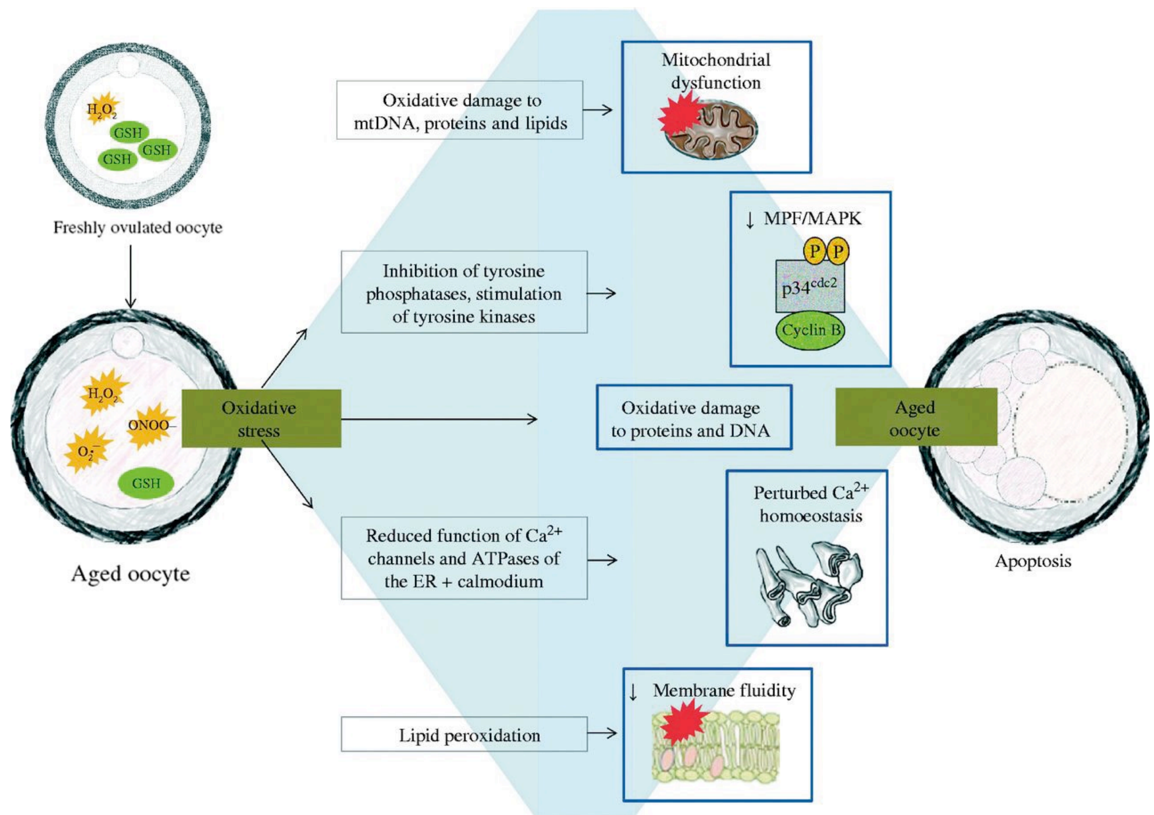

Figure 2 The post-ovulatory oocyte ageing process via multiple pathways (Source: Lord & Aitken, 2013)

1.5.1. Influence of ROS on MPF

Lord & Aitken (2013) proposed that the decline in the critical cell cycle factor, MPF, could be a symptom of oxidative stress in the MII oocyte. As discussed previously, during post-ovulatory ageing the catalytic subunit of MPF is phosphorylated at multiple sites which leads to the accumulation of the inactivated compound “pre-MPF”. Two enzymes, cdc25 (a tyrosine phosphatase) and Wee1/Myt1 (tyrosine kinases) purportedly control these phosphorylation events (Kikuchi et al., 2002). ROS have previously been shown to have the capacity for inhibition of tyrosine phosphatases, including cdc25 specifically and stimulation of tyrosine kinases (Lord & Aitken, 2013). Thus, ROS induced oxidative stress could directly affect MPF levels within the cell, resulting in the induction of parthenogenetic activation and fragmentation (Kikuchi et al., 2002).

1.5.2. ROS-induced Lipid Oxidation

A significant decrease in several phospholipid classes was reported both in hydrogen peroxide-treated and aged oocytes (Mok et al., 2016), while over-ripening of rainbow trout eggs was accompanied by an increase in free lipid and a decrease in lipid phosphorus, as measures of phospholipids (Craik & Harvey, 1984). In a mouse model, Takahashi et al., (2003) reported that lipid peroxidation, an indicator of the degree of oxidative stress, in the membranes of *in vivo* aged oocytes was higher than seen in fresh oocytes. Following lipid

peroxidation in the oocyte plasma membrane, a reduction in membrane fluidity is observed. This could decrease the oocyte's potential for sperm-oocyte fusion, resulting in a decreased fertilization rate in aged oocytes (Lord & Aitken, 2013).

1.5.3. ROS-induced Mitochondrial Dysfunction

The number and function of mitochondria in oocytes declines during maternal ageing (Simsek-Duran et al., 2013). Postovulatory and maternal ageing of oocytes both involve altered redox state and mitochondrial function defects (Takahashi et al., 2009). Studies on higher vertebrates have indicated that ROS-induced oxidative stress in post-ovulatory aged oocytes significantly disrupts mitochondrial function (Takahashi et al., 2003). It has been proposed that this damage is more severe than that observed in maternally aged oocytes (Samarin et al., 2019a).

In 1995 based on the "oxygen radical-mitochondrial injury hypothesis of ageing" (Miquel et al., 1980) Tarín proposed a mechanism to explain the effects of oocyte ageing on subsequent viability and potential for embryo and fetal development. In this mechanism oxygen radical damage to mitochondrial DNA, proteins and lipids plays a key role in the ageing process of the oocyte (Tarín, 1995). Oxidative damage to mitochondria may both decrease the number of functionally intact mitochondria and thereby decrease intracellular ATP concentration as well as raise the production of reactive oxygen species by the electron transport chain (Tarín et al., 2000).

The DNA, proteins and lipids within the mitochondria are not only close to the source of ROS production, the electron transport chain, but the mitochondrial DNA lacks the protective histones and mechanisms for DNA repair. This makes mitochondria particularly susceptible to oxidative attack (Lord & Aitken, 2013). Since Tarín postulated his hypothesis, loss of mitochondrial membrane potential (Liu et al., 2000), increased ROS generation by the electron transport chain (Liu et al., 2009a), damage and deletions to mtDNA, and a decline in ATP production have all been linked to oxidative stress (Lord & Aitken, 2013). Furthermore, increase in ROS generation and a decline of ATP production in the mitochondria have been linked to postovulatory ageing (Lord & Aitken, 2013).

1.5.4. Disturbance in Intracellular Ca^{2+} Regulation

ATP produced in mitochondria is necessary for many processes in oocytes (Samarin et al., 2019a), including Ca^{2+} oscillations at fertilization as well as Ca^{2+} homeostasis in oocytes (Dumollard et al., 2007). The increase in Ca^{2+} concentration plays important roles in fertilization and subsequent embryo development (Takahashi et al., 2013).

In mammalian oocytes undergoing fertilization, sperm induces drastic changes in the intracellular Ca^{2+} concentration consisting of a single long-lasting rise in Ca^{2+} concentration followed by short repetitive changes in Ca^{2+} concentration that last for several hours. These changes in the concentration of Ca^{2+} over timer are called "Ca^{2+} oscillations" (Takahashi et al., 2013). Ca^{2+} oscillations convey important information about the development of oocytes (Fissore et al., 2002) and alterations in the oscillation pattern of Ca^{2+} concentration affects the early and post-implantation embryo development (Takahashi et al., 2013).

At fertilization, abnormal oscillations of Ca^{2+} were observed in both *in vivo*- and *in vitro*-aged oocytes. The oscillation of Ca^{2+} levels exhibited a higher frequency but lower amplitude in more aged oocytes than in fresh oocytes. When fresh oocytes were treated with hydrogen peroxide the Ca^{2+} oscillation pattern was similar to that seen in aged oocytes (Takahashi et al., 2003).

The Ca^{2+} stores within the endoplasmic reticulum (ER) are replenished from the cytosol through the activity of the smooth endoplasmic reticulum Ca^{2+}-ATPases (SERCA). The activity of SERCA is highly dependent on the availability of intracellular ATP (Takahashi et al., 2013). Both *in vivo*- and *in vitro*-postovulatory-aged oocytes have decreased ATP content in unfertilized and fertilized mouse oocytes (Igarashi et al., 2005), which could be the cause of impaired reuptake of Ca^{2+} by SERCA in the ER in aged oocytes (Igarashi et al., 1997). Due to depletion of Ca^{2+} stores in the ER, the release of Ca^{2+} from 1,4,5-triphosphate (InsP3)-sensitive Ca^{2+} stores is diminished in both *in vivo*- and *in vitro*- aged oocytes (Takahashi et al., 2009). Taken together, these results indicate an impaired Ca^{2+} homeostasis in postovulatory aged oocytes (Takahashi et al., 2013).

Additionally, it has been reported that ROS can directly affect Ca^{2+} channels and Ca^{2+}-ATPases of the endoplasmic reticulum (Wesson & Elliot, 1995), as well as the Ca^{2+} signaling molecule calmodulin (Gao et al., 2001).

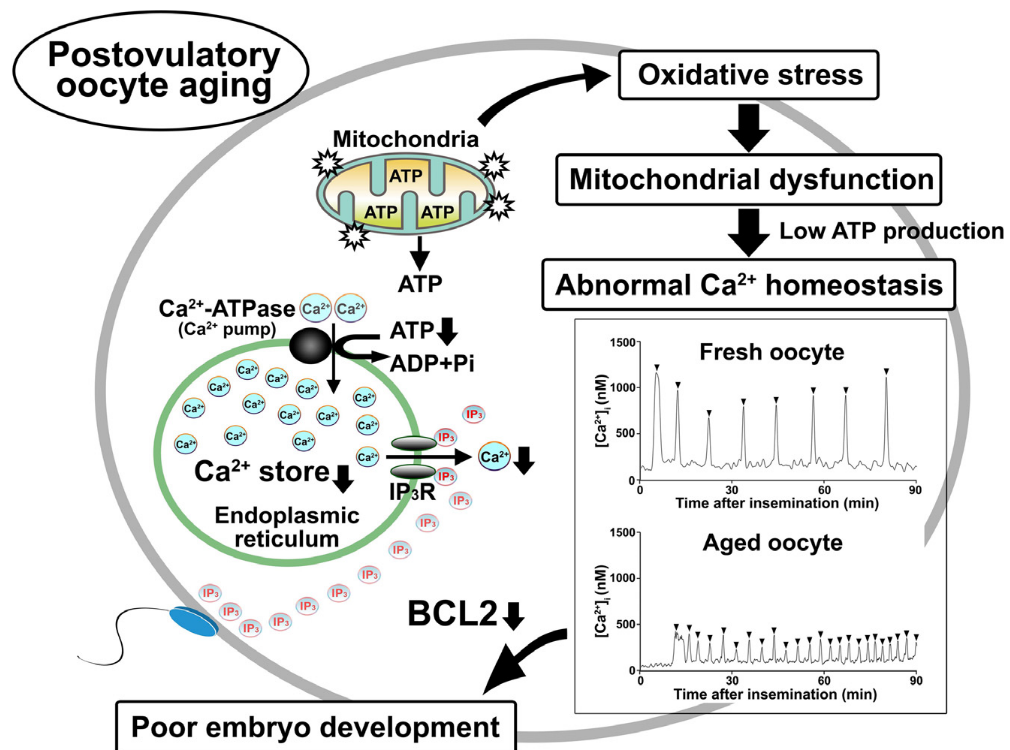

Figure 3 Schematic of the mechanism of poor embryo development in postovulatory oocyte aging (Source: Takahashi et al., 2013)

1.5.5. ROS-induced Damage to DNA and Proteins

Oxidative stress is also capable of generating global damage to DNA and proteins (Lord et al., 2013). The treatment of rat oocytes with H_2O_2 caused DNA fragmentation (Chaube et al., 2005). In proteins, oxidative stress increases the rate of disulfide bond and mixed disulfide formation which can lead to inactivation of some enzymes (Guérin et al., 2001). Poor embryo quality could be the consequence of such global acquisition of oxidative damage in aged oocytes, as relief from oxidative stress in the aged MII oocyte has been linked with improved developmental potential (Lord et al., 2013).

1.6. Mechanism for Preventing or Delaying Oxidative Stress

1.6.1. Culture Conditions and Media Composition

Changes in the concentration of metabolites within oocyte culture media has been shown to prominently affect post-ovulatory ageing *in vitro* (Lord & Aitken, 2013). Increasing the concentration of pyruvate has been shown to delay post ovulatory ageing and delaying the onset of apoptosis (Liu et al., 2009b). It has been theorized that, since the oocyte and early embryo preferentially utilize pyruvate over glucose to drive metabolism (Lord & Aitken, 2013) pyruvate supplementation delays oocyte ageing by fueling prolonged ATP production within oocytes as well as by maintaining intracellular redox potential (Liu et al., 2009b).

Changes in the temperature of the culture have also been shown to delay the onset of post-ovulatory ageing *in vitro* (Lord & Aitken, 2013). Egg viability studies in the fish species

Rutilus frisii kutum showed that the egg viability of *in vitro* stored oocytes decreases with increased storage temperature. Eggs stored at 4 °C could be successfully fertilized and maintain developmental potential after 8 h post-stripping, whereas oocytes stored at 26 °C lost their viability almost completely after 4 HPS (Samarin et al., 2011). The delayed ageing at low temperature is likely related to a down-regulation of oocyte metabolism (Lord & Aitken, 2013) which results in reduced ROS production and, a subsequent decrease in the likelihood of oxidative damage and ROS-induced apoptosis (Li et al., 2012).

1.6.2. Maintaining MPF Levels

Delaying or preventing post-ovulatory oocyte ageing *in vitro* by maintaining levels of critical cell cycle factors within the cell has been thoroughly investigated (Lord & Aitken, 2013). Supplementation of oocyte culture media with caffeine has been shown to maintain levels of MPF post-ovulation, as caffeine acts to inhibit phosphorylation of MPF to pre-MPF and thus subsequently decreasing levels of parthenogenesis and fragmentation (Kikuchi et al., 2002).

However, caffeine is an inhibitor of DNA repair mechanisms, and the impairment of DNA repair has been demonstrated to directly affect oocytes (Lord & Aitken 2013). While oocytes that were treated with caffeine have produced live offspring (Ono et al., 2011), there is clearly a concern about the safety of utilizing caffeine as an agent for preventing post-ovulatory oocyte ageing *in vitro*, as un-repaired DNA within the embryo may lead to abnormalities and/or birth defects in offspring (Lord & Aitken, 2013).

However, not all aspects of post-ovulatory ageing can be controlled by manipulation of cell cycle factors. Caffeine-supplemented oocytes show no delay in the onset of age-associated apoptosis, nor any relief from accumulation of oxidative stress (Lord et al., 2013).

1.6.3. Antioxidants

If oxidative stress acts as the initiator for post-ovulatory ageing, then relief from oxidative stress by way of antioxidant supplementation should prevent or delay the ageing process (Samarin et al., 2019a).

Insemination of *in vitro* aged mouse oocytes in the presence of different concentrations of antioxidants like L-ascorbic acid, 6-methoxy-2,5,7,8-tetramethylchlormane-2-carboxylic acid (Trolox) and ethylenediaminetetraacetic acid (EDTA) could not prevent cellular fragmentation or improve the fertilization rate associated with post-ovulatory ageing. However, the addition of the reducing agent dithiothreitol (DTT) to oocyte culture medium resulted in increased fertilization and blastocyst formation rates. This demonstrates that that the negative effects of post-ovulatory oocyte ageing on reproductive potential can be reduced

with antioxidant therapy *in vitro*. However, the results depended on the type of antioxidant utilized (Tarín et al., 1998).

Supplemented to *in vitro* cultures the antioxidant melatonin has been shown to reduce levels of ROS by 41% thus relieving ageing mouse oocytes of oxidative stress, delaying the onset of apoptosis and preventing fragmentation. Melatonin-supplemented oocytes experienced an increased optimal window for fertilization and improved embryo quality when compared with control aged oocyte counterparts (Lord et al., 2013). Earlier studies highlighted the ability of melatonin to reverse the damaging effects of H_2O_2 treatment on MII oocytes (Tamura et al., 2008). Melatonin is a primary candidate for utilization in an assisted reproduction setting as it lacks any demonstrable toxicity (Lord & Aitken, 2013).

Antioxidants may be at the forefront in terms of delaying *in vitro* ageing prior to in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) but their ability to influence to delay *in vivo* post-ovulatory ageing is poorly defined. Reports of oral administration of antioxidants focus primarily on the relationship between antioxidants and ovarian ageing. Following the success of antioxidant supplementation *in vitro*, it would be beneficial to establish the capability of these compounds to delay the onset of senescence in MII oocytes *in vivo* (Lord & Aitken, 2013).

1.7. *Clarias gariepinus*

Catfishes are a large group of predominantly freshwater fishes that belong to the *Siluriformes* order (Teugels & Gourene, 1998). Within the *Clariidae* family, the *Clarias* genus is the most numerous group containing at least 61 species spread over Asia and Africa (Maneechot et al., 2016). This genus contains the most cultured African catfish *Clarias gariepinus* (Teugels & Gourene, 1998).

The African catfish (*Clarias gariepinus*) is widely distributed in Africa and Parts of Asia. It primarily habituates in calm lakes, rivers and swamps as well as a seasonal floodplain. As adaptations to life in stagnant environments, *C. gariepinus* feature pseudo-lungs, long bodies and a high capacity to produce mucous. It reproduces seasonally, with gonadal maturation that coincides with periods of floods. Changes in water temperature and photoperiod impact the maturation process, but the increase in water level is the most important factor in their reproduction (Vitule te al., 2006).

The African catfish is an opportunistic and omnivorous feeder that ingests algae, macrophytes, zooplankton, insects, fish prey, detritus, Amphibians and sand grains. The diet composition may differ depending on the season, environment as well as the size and maturity of the fish (Tesfahun, 2018).

The selection of *C. gariepinus* for industrial aquaculture production was favored by its large size (up to 1.5 m), omnivorous feeding habits, Pan-African distribution and its capacity to survive in poorly oxygenated water (Teugels & Gourene, 1998). Due to its ability to spawn several times during the season African catfish has good potential not only for commercial fish production, but also for commercial caviar production. The primary constraint for such production is the African catfish's inability to spawn in artificial environment, which means that breeding must be artificially induced (Romanova et al., 2018). Despite this, relatively lesser production costs, lesser environmental control requirements for breeding as well as the excellent quality of the meat as a protein source make *C. gariepinus* a viable aquaculture endeavor (Kucharczyk et al., 2019).

The global production of *C. gariepinus* increased from about 5000 metric tons in 1992 to 248,000 metric tons in 2015. A further increase in production growth is expected especially given that African catfish are a warm water species that can be effectively cultivated on a large scale across continents (Kucharczyk et al., 2019).

2. Aims of the Work

- To investigate the extent of lipid oxidation in the oocytes of African catfish *Clarias gariepinus* by measuring possible changes in the levels of TBARS at varying ageing times *in vitro* up to the egg over-ripening stage and complete loss of fertilizing ability.
- To investigate the extent of protein oxidation in the oocytes of African catfish *Clarias gariepinus* by measuring possible changes in the levels of carbonyls at varying ageing times *in vitro* up to the egg over-ripening stage and complete loss of fertilizing ability.
- To analyze the correlation of oocyte lipid and protein oxidation content with the success of fertilization.

3. Materials and Methods

The oxidation products during in vitro oocyte aging in African catfish was assessed by three experiments as: (I) egg storage and quality assessment, (II) lipid oxidation and (III) protein oxidation assay.

3.1. Egg Storage and Quality Assessment

The in vitro egg storage and the embryo quality assessment were done as bellow:

3.1.1. Experimental Fish Preparation

The broodfish preparation and artificial reproduction processes were performed according to Samarin et al. (2018b). Briefly, the experimental fish were treated with a gradual increase in water temperature to 23 °C. Ovulation was induced with Ovopel preparation (Interfish Ltd., Hungary) (dosage 20 lg of GnRHa + 2 mg of metoclopramide per 1 kg of body weight when 1 pellet was dissolved in 1 ml of saline solution and applied via intramuscular injection). The experiment was conducted using five females. Male brood fish were not treated hormonally. To obtain testicular sperm required for artificial fertilization, males were manually sacrificed, and the dissected testes stored at 10 °C.

3.1.2. In Vitro Oocyte Aging

Oocytes from females were individually incubated *in vitro* for 16 h post-stripping (HPS) at 25 °C. The stored oocytes were fertilized at the time of stripping (0 HPS) and then at 2, 4, 8 and 16 HPS. *In vitro* egg storage conditions were determined according to Samarin et al. (2018b).

3.1.3. Artificial Fertilization and Egg Developmental Success

Embryo survival and hatching rates were recorded as the egg quality indices. Embryo survival rates were measured 24 h after fertilization using a stereomicroscope (Nikon SMZ745T, Japan). Hatching rates were calculated 48 h after fertilization by counting the number of embryos that reached the hatched-larvae stage to the number of initially fertilized eggs. All steps for artificial fertilization, removing egg stickiness, incubation, and assessment of egg developmental success were performed according to Samarin et al. (2018b).

3.1.4. Sample Collection for Analysis

Approximately 1 g of oocytes was sampled from individual females separately at various timepoints from spawning until oocyte over-ripening occurred. The collected samples were placed into cryotubes, labelled, frozen in liquid nitrogen, and stored at -80 °C for further investigation of oxidative products.

3.2. Lipid Oxidation

Lipid peroxidation is a process in which oxidants such as free radicals or nonradical species attack lipids containing carbon-carbon double bond(s) like polyunsaturated fatty acids. The process involves the abstraction of a hydrogen from a carbon and insertion of an oxygen molecule (Ayala et al., 2014) resulting in a mixture of complex products including lipid peroxyl radicals and hydroperoxides as the primary products, as well as malondialdehyde (MDA) and 4-hydroxynonenal as predominant secondary products (De Leon & Borges, 2020).

MDA is considered as the main marker in lipid peroxidation (Zeb & Ullah, 2016) and has widely been used in biomedical research (De Leon & Borges, 2020). Thiobarbituric acid (TBA) is reacted with MDA, which leads to the formation of MDA-TBA2, a red-pink conjugate that absorbs in the visible spectrum at 532 nm and can be determined spectrophotometrically, chromatographically, or through image processing techniques (Zeb & Ullah, 2016). However, other compounds generated from lipid peroxidation, in addition to MDA, can also react with TBA and absorb light at 532 nm, adding to the overall absorption signal that is measured. Because of this, the assay is considered to measure "thiobarbituric acid reactive substances" or TBARS. Additionally, MDA has the ability to react with most other major classes of biomolecules, which can potentially restrict its accessibility for reaction with TBA. Despite this, when correctly applied and interpreted, the TBARS assay is considered a good indicator of the overall levels of oxidative stress in a biological sample (De Leon & Borges, 2020).

The thiobarbituric acid reactive substances (TBARS) method was used to evaluate oocyte lipid peroxidation according to Li et al. (2010). 800 µL of 10 % TCA in 0.2 M phosphoric acid and 200 µL of BHT (0.09 mol L^{-1}) in methanol were added to 0.2 g of fish oocytes and afterwards homogenized by sonication using UltraTurrax (Janke & Kunkel, Staufen, Germany, T25IKA-Labortechnik) in three 20-second intervals at a speed of approximately 14 000 rpm. After centrifugation at 15 000 rpm for 10 min at 4 °C the volume of the supernatant was divided into two clean Eppendorf tubes, 300 µL of the supernatant were added into each tube. To one of the duplicates, the blank, 300 µL of double-distilled water was added while to the other 300 µL of TBA (0.02 M) were added.

For calibration 400 µL of 10 % TCA in 0.2 M phosphoric acid was mixed with 400 µL double-distilled water. In clean Eppendorf tubes: 0, 10, 20, 50, 100, 200, 400 and 500 µL of 25 µM TEP were pipetted, this was repeated two times. Subsequently, one set of tubes was filled up to 500 µL with 10 % TCA in 0.2 M phosphoric acid and the other with double distilled

water. 500 µL of TBA (0.02 M) were added to all of the tubes. The tubes were vortexed well. All of the tubes were incubated for 45 min in an 85 °C water bath.

Each sample was pipetted 3 times to a 96-well microplate, 150 µL per well. The absorbance of the samples was read at wavelength of 530 nm against the sample blank using a UV-visual plate reader (AF 2200; Austria). Results were expressed as equivalents to malonaldehyde (MDA) in Lg/g.

3.3. Protein Oxidation

A type of protein oxidation that can be promoted by ROS is protein carbonylation. In this process, reactive carbonyl groups (aldehydes and ketones) are produced on protein side chains. In ''primary protein carbonylation'' direct oxidation of amino acid side chains occurs, but carbonyl groups can also be introduced into proteins by ''secondary protein carbonylation'' where nucleophilic side chains of proteins react with aldehydes produced during lipid peroxidation (Suzuki et al., 2010). This makes protein carbonyl content the most general indicator and by far the most commonly used marker of protein oxidation. The moieties that form are chemically stable, which is useful for their detection (Dalle-Donne et al., 2003).

In a highly sensitive assay, the carbonyl group can be reacted with 2,4-dinitrophenylhydrazine (DNPH) which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product which can be detected by various means. Since the DNP group itself absorbs ultra-violet light at 370 nm, the total carbonyl content of a protein or mixture of proteins can be quantified by a spectrophotometric assay (Dalle-Donne et al., 2003).

Protein oxidation was estimated as carbonyls formed after incubation with 2,4-dinitrophenylhydrazine (DNPH) in 2 N hydrochloric acid following a slightly modified method from the one described by Oliver et al. (1987). 800 µL of KCl (0.15 M) were added to 0.2 g of fish eggs and afterwards homogenized by sonication using UltraTurrax (Janke & Kunkel, Staufen, Germany, T25IKA-Labortechnik) in three 20-second intervals at a speed of approximately 14000 rpm. 100 µL duplicates of the homogenized solution were transferred to 2 mL Eppendorf tubes. Each tube was vortexed prior to precipitation with 1 mL of TCA (10 %). After centrifugation at 5000 rpm for 5 min the supernatant was removed. To one of the duplicates, the blank, 1 mL of HCl (2 N) was added while to the other 1 mL of 0.2 % of DNPH in HCl (2 N) was added. The samples were left at ambient temperature for one hour and were shaken and vortexed every 20 minutes. The sample was then reprecipitated with 1 mL of TCA (10%), vortexed and centrifuged for 5 min at 5000 rpm. Afterwards, the supernatant was removed. 1 mL of ethanol-ethyl acetate (1:1) was added to the pellets and centrifuged for 5

minutes at 10 000 rpm. The pallets were washed with ethanol-ethyl acetate (1:1) twice more. Then, 1.5 mL of 6 M guanidine hydrochloride with 20 mM sodium phosphate buffer, pH 6.49 were added onto the precipitate, vortexed, homogenized using the UltraTurrax high-performance dispersing machine and centrifuged for 2 min at 5000 rpm.

The supernatant was transferred to a 96-well microplate and read in the UV-visual plate reader (AF 2200; Austria) at 280 nm and 370 nm. The protein content was measured at 280 nm and the concentration was determined by Bradford reagent using a 6 M BSA in guanidine with sodium phosphate buffer as standard protein. Carbonyl concertation was calculated based on the millimolar absorptivity of aliphatic hydrazones most of which have the extinction molar coefficient of 21.0 mM^{-1} cm^{-1} at 370 nm.

3.4. Statistical Analysis

The normality of the data was assessed using SPSS statistical software (version 18-IBM Corp) for Windows. Differences between the means of the groups (different HPSs) for the embryo survival rates, hatching rates as well as the lipid and protein oxidation among the different HPSs were evaluated using ANOVA followed by Duncan's multiple range test. $P < .05$ was considered significant.

3.5. Principles of Other Methods

Sonication refers to the process of applying sound energy to agitate particles in a sample. Since usually ultrasonic frequencies (>20 kHz) are used, the process is also known as ultrasonication. Homogenization occurs both directly through the ultrasonic forces as well as through cavitation – the rapid formation and violent collapse of microscopic bubbles. The combination of ultrasonic forces and cavitation makes ultrasonic homogenizers excellent for cell disruption and particle size reduction. Sonication can be conducted directly where a probe is inserted directly into the sample vessel or indirectly with the use of an ultrasonic bath.

Ultraviolet-visible (UV/Vis) spectroscopy uses light in the ultraviolet and visible spectral regions to obtain quantitative or qualitative information about an analyte. When the frequency of the incoming light is equal to the energy difference between a molecule's ground and excited states absorption occurs. A UV/Vis spectrophotometer measures the intensity of light transmitted through a sample compared to a reference measurement of the incident light source.

4. Results

4.1. Egg Quality Indices

The embryo survival and hatching rates were significantly affected by post-stripping oocyte aging, as presented in Table 1. A complete loss of egg viability occurred at 16 hours post-stripping (HPS) when eggs were stored at 25 °C. Embryo mortality and larval malformation rates increased significantly over time and were the highest in the most aged oocytes. When eggs were stored at 25 °C, 94 % of the embryos died before hatching and 50 % of the hatched larvae were malformed at 8 HPS (Table 1).

Table 1 Effects of post-stripping ova ageing on the embryo survival, hatching, embryo mortality and larval malformation rates of African catfish eggs stored at 25 °C (mean ± SD). Means sharing a common alphabetical symbol do not differ significantly among the different HPS under the investigated parameters.

In Vitro Oocyte Aging					
HPS	**0**	**2**	**4**	**8**	**16**
Embryo survival %	88 ± 3.8 ^{a}	80 ± 7.3 ^{ab}	58 ± 1.1 ^{b}	24 ± 1,9 ^{c}	0 ± 0 ^{d}
Hatching %	81 ± 5.3 ^{a}	63 ± 0.7 ^{b}	35 ± 1.5 ^{c}	2 ± 3.8 ^{d}	0 ± 0 ^{d}
Embryo mortality %	8 ± 2.1 ^{a}	21 ± 7.8 ^{a}	40 ± 1.3 ^{b}	94 ± 1.2 ^{c}	--------
Larval malformation %	5 ± 3.4 ^{a}	12 ± 1.9 ^{ab}	17 ± 9.3 ^{b}	50 ± 11.8 ^{c}	---------

4.2. Lipid Oxidation during *In Vitro* Oocyte Ageing

The level of MDA as an indicator of oxidative stress exhibited no significant change during post-stripping ageing (Figure 1).

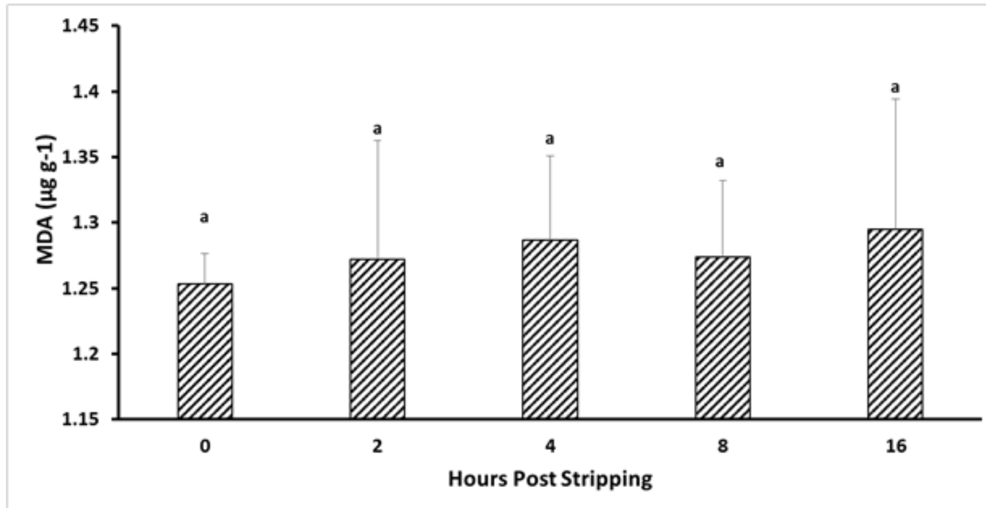

Figure 4 Effect of *in vitro* oocyte ageing at 25°C in African catfish on TBARS, expressed as malonaldehyde (µg/g)

4.3. Protein Oxidation during *In Vitro* Oocyte Ageing

Protein oxidation, measured as carbonyl values, did not change significantly, and remained constant at around 6 nmol/mg (Figure 2).

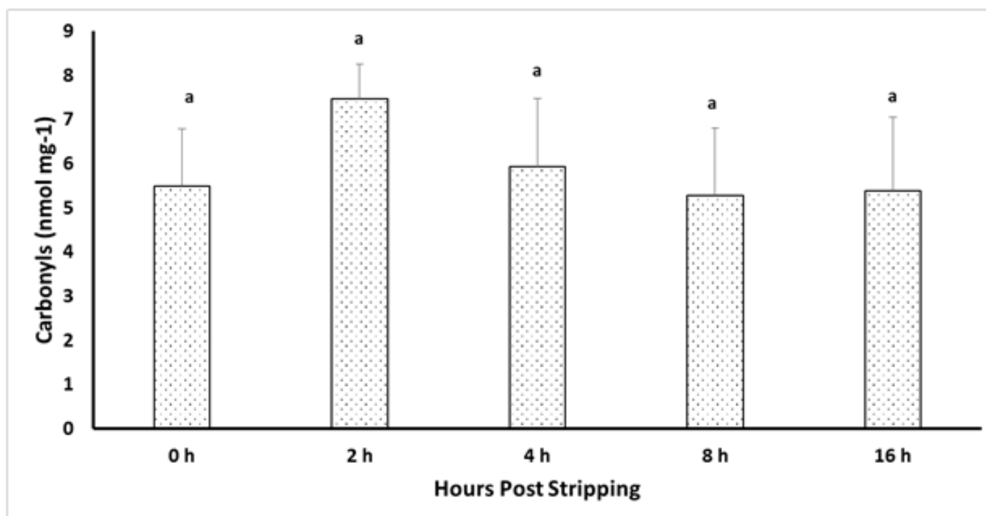

Figure 5 Effect of *in vitro* oocyte ageing at 25 °C in African catfish on carbonyls (nmol/mg)

5. Discussion

Post-ovulatory ageing is known to reduce the oocyte quality and can lead to defects in emerged embryo developmental competence. Additionally increasing time between ovulation and fertilization enhances cytogenetic abnormalities (Petri et al., 2020) and leads to unhealthy status in later life of the offspring (Tarín et al., 2002). Hence, interests are rising to investigate the poorly understood mechanisms underlying these consequences in aged oocytes. Samarin et al. (2018b) showed that during *in vitro* storage of African catfish oocytes at 25 °C, the highest embryo survival, and hatching rates (88% and 80%, respectively) were obtained from eggs that were fertilized immediately after stripping. The embryo survival rates remained constant, at around 80%, for the eggs fertilized up to 2 hours post-stripping (Table 1). Then, the survival percentages decreased significantly by prolonging the time storage and were 24% for the eggs stored in vitro for 8 hours post stripping (HPS). After 16 hours of oocyte storage, the fertilization rate was totally lost, and no viable embryos were detected. Hatching rates significantly and steadily decreased over the entire ageing time. The embryo mortality and larval malformation rates did not show any marked increase during 2 hours of *in vitro* egg storage. After 2 HPS, the embryo mortality and larval malformations increased significantly over time so that at 8 HPS, 94% of the embryos died before hatching and 50% of the hatched larvae were malformed (Table 1). The detailed results of egg viability success can be referred in Samarin et al. (2018b).

Ageing of oocytes certainly occurs post-ovulation and leads to decreased fertilization potential. However, the precise molecular mechanisms underlying post-ovulatory oocyte ageing and its deleterious effects are still unclear in fish and in other vertebrates. Various theories have been raised to find the actual path of oocyte ageing. Oxidative stress is proposed as a possible initiator of a cascade of events that occur during the oocyte ageing process in other vertebrates (Lord & Aitken, 2013, Takahashi et al., 2013, Lord et al., 2013). Mitochondrial oxidative phosphorylation is the primary source of energy in the oocyte (Dumollard et al., 2007). This oxidative phosphorylation produces most of the cellular reactive oxygen species (ROS) as a by-product. Furthermore, other environmental factors like exposure to light, lack of antioxidants, and increased oxygen lead to oxidative stress in post-ovulatory aged oocytes (Lord & Aitken, 2013). Oxidative stress during post-ovulatory oocyte ageing deteriorates the fertilizing ability of the oocyte due to its effect on several cellular fundamental components like DNA and proteins (Lord & Aitken, 2013, Homer, 2021, Wang et al., 2021). It has been reported in a mouse model of oocyte ageing that lipid peroxidation in the membrane of *in vivo* aged oocytes is higher than that in fresh oocytes (Takahashi et al.,

2003) which suggests the involvement of oxidative stress in the unfavourable outcomes of oocyte ageing.

The present study examined whether oxidative stress affects the progress of oocyte ageing, as has been proposed for higher vertebrates. This was done by investigating the oxidation status of African catfish oocytes during post-stripping ageing. TBARS as a marker of lipid oxidation and carbonyls, which show the extent of protein oxidation were measured based on spectrophotometric assays. The level of TBARS did not change significantly and remained at around 1.3 μg g^{-1} malondialdehyde (MDA) during the progress of oocyte ageing (Figure 1). Similarly, carbonyl values, stayed constant through 16 HPS (Figure 2). Samarin et al. (2019c) investigated the effects of both *in vivo* and *in vitro* oocyte ageing in common carp on TBARS and carbonyls. They reported that with elapsing time following ovulation the amount of TBARS which is the main indicator of lipid oxidation and the amounts of carbonyls which show the extension of protein oxidation did not change in the oocytes and indicated no increase in oxidative stress (Samarin et al., 2019c). In addition, in goldfish oocytes the amount of thiobarbituric acid reactive substances (TBARS), did not change over time following stripping (Samarin et al., 2019b). Oxidative lipid damage was not observed in tench oocytes during oocyte ageing when indicated by MDA levels however, tench oocytes demonstrated significant age-related decrease in protein oxidation (Samarin et al., 2018a).

Transcriptomic analyses revealed no differences in the mRNA abundance of the selected genes related to oxidative stress in African catfish *Clarias gariepinus* (Samarin et al., 2018b). Additionally, the mRNA abundance of selected genes involved in oxidative injury and stress responses did not show significant changes during post ovulatory oocyte ageing in common carp (Samarin et al., 2019c) and goldfish (Samarin et al., 2019b). These authors concluded that it is unlikely that the oxidative stress is the initiator of the oocyte ageing process, but most probably appears in the later stages because of post-ovulatory ageing (Samarin, 2018). Antioxidant enzyme analysis in previous studies indicated no significant changes in the activity of catalase and superoxide dismutase during the post-ovulatory ageing of common carp (Samarin et al., 2019c), goldfish (Samarin et al., 2019b), and tench oocytes (Samarin et al., 2018a). In the present study, the biochemical indicators of lipid peroxidation and protein oxidation didn't show significant changes during oocyte ageing in African catfish which indicates no involvement of oxidative stress during African catfish oocyte ageing at least until the complete loss of egg fertilizing ability. Additional tests and analysis, however, are needed to entirely investigate the role of oxidative stress in defect of egg quality during oocyte ageing.

6. Conclusion

Considerable defects of egg fertilizing ability and developmental success are observed during African catfish oocyte ageing. African catfish *Clarias gariepinu* oocytes stored at 25 °C completely lose their viability at 16 hours post-stripping (HPS). Postovulatory ageing of African catfish oocytes for up to 16 hours after oocyte ovulation exhibited no effect on lipid oxidation. Comparison of the protein oxidation status between freshly ovulated and aged oocytes revealed constant values for carbonyls. The results of the current study suggest no involvement of oxidative stress during African catfish oocyte ageing at least until the complete loss of egg fertilizing ability. For the future it would be interesting to investigate whether post-ovulatory oocyte ageing induces oxidative stress in resulting embryos from differently aged oocytes.

7. References

Aegerter, S., & Jalabert, B. (2004). Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, Oncorhynchus mykiss. *Aquaculture*, *231*(1-4), 59-71.

Agarwal, A., & Prabakaran, S. A. (2005). Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. *Indian Journal of Experimental Biology, 43*, 963-974

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative medicine and cellular longevity*, *2014*.

Bobe, J., & Labbé, C. (2010). Egg and sperm quality in fish. *General and comparative endocrinology*, *165*(3), 535-548.

Boerjan, M. L., & De Boer, P. (1990). First cell cycle of zygotes of the mouse derived from oocytes aged postovulation in vivo and fertilized in vivo. *Molecular reproduction and development*, *25*(2), 155-163.

Brooks, S., Tyler, C. R., & Sumpter, J. P. (1997). Egg quality in fish: what makes a good egg? *Reviews in Fish Biology and fisheries*, *7*(4), 387-416.

Chaube, S. K., Prasad, P. V., Thakur, S. C., & Shrivastav, T. G. (2005). Hydrogen peroxide modulates meiotic cell cycle and induces morphological features characteristic of apoptosis in rat oocytes cultured in vitro. *Apoptosis*, *10*(4), 863-874.

Chi, M. M. Y., Manchester, J. K., Yang, V. C., Curato, A. D., Strickler, R. C., & Lowry, O. H. (1988). Contrast in levels of metabolic enzymes in human and mouse ova. *Biology of reproduction*, *39*(2), 295-307.

Craik, J. C. A., & Harvey, S. M. (1984). Egg quality in rainbow trout: the relation between egg viability, selected aspects of egg composition, and time of stripping. *Aquaculture*, *40*(2), 115-134.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica chimica acta*, *329*(1-2), 23-38.

De Leon, J. A. D., & Borges, C. R. (2020). Evaluation of oxidative stress in biological samples using the thiobarbituric acid reactive substances assay. *Journal of Visualized Experiments*, (159), e61122.

Dumollard, R., Ward, Z., Carroll, J., & Duchen, M. R. (2007). Regulation of redox metabolism in the mouse oocyte and embryo. *Development*, *134*, 455-465.

Fan, H. Y., & Sun, Q. Y. (2004). Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biology of reproduction*, *70*(3), 535-547.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*(6809), 239-247.

Fissore, R. A., Kurokawa, M., Knott, J., Zhang, M., & Smyth, J. (2002). Mechanisms underlying oocyte activation and postovulatory ageing. *Reproduction, 124*(6), 745-754.

Gao, J., Yao, Y., & Squier, T. C. (2001). Oxidatively modified calmodulin binds to the plasma membrane Ca-ATPase in a nonproductive and conformationally disordered complex. *Biophysical journal*, *80*(4), 1791-1801.

Goud, A. P., Goud, P. T., Diamond, M. P., Gonik, B., & Abu-Soud, H. M. (2008). Reactive oxygen species and oocyte aging: role of superoxide, hydrogen peroxide, and hypochlorous acid. *Free Radical Biology and Medicine*, *44*(7), 1295-1304.

Guérin, P., El Mouatassim, S., & Menezo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human reproduction update*, *7*(2), 175-189.

Homer, H. A. (2021). Understanding oocyte ageing: can we influence the process as clinicians?. *Current Opinion in Obstetrics and Gynecology*, *33*(3), 218-224.

Igarashi, H., Takahashi, E., Hiroi, M., & Doi, K. (1997). Aging-related changes in calcium oscillations in fertilized mouse oocytes. *Molecular Reproduction and Development: Incorporating Gamete Research*, *48*(3), 383-390.

Igarashi, H., Takahashi, T., Takahashi, E., Tezuka, N., Nakahara, K., Takahashi, K., & Kurachi, H. (2005). Aged mouse oocytes fail to readjust intracellular adenosine triphosphates at fertilization. *Biology of reproduction*, *72*(5), 1256-1261.

Kikuchi, K., Naito, K., Noguchi, J., Kaneko, H., & Tojo, H. (2002). Maturation/M-phase promoting factor regulates aging of porcine oocytes matured in vitro. *Cloning & Stem Cells*, *4*(3), 211-222.

Kotani, T., & Yamashita, M. (2002). Discrimination of the roles of MPF and MAP kinase in morphological changes that occur during oocyte maturation. *Developmental biology*, *252*(2), 271-286.

Kucharczyk, D., Kucharczyk, D. J., Nowosad, J., & Omirzhanova, N. (2019). Optimization of artificial insemination outcomes of African catfish (Clarias gariepinus) with differing hatchery conditions. *Animal reproduction science*, *211*, 106222.

Li, P., Li, Z. H., Dzyuba, B., Hulak, M., Rodina, M., & Linhart, O. (2010). Evaluating the impacts of osmotic and oxidative stress on common carp (Cyprinus carpio, L.) sperm caused by cryopreservation techniques. *Biology of reproduction*, *83*(5), 852-858.

Li, Q., Wang, G., Zhang, J., Zhou, P., Wang, T. Y., Cui, W., Luo, M. J., & Tan, J. H. (2012). Combined inhibitory effects of pyruvate and low temperature on postovulatory aging of mouse oocytes. *Biology of reproduction*, *87*(5), 105-1.

Li, R., Jia, Z., & Trush, M. A. (2016). Defining ROS in biology and medicine. *Reactive oxygen species (Apex, NC)*, *1*(1), 9.

Liu, H., Wang, T., & Huang, K. (2009a). Cholestane-3β, 5α, 6β-triol-induced reactive oxygen species production promotes mitochondrial dysfunction in isolated mice liver mitochondria. *Chemico-biological interactions*, *179*(2-3), 81-87.

Liu, L., Trimarchi, J. R., & Keefe, D. L. (2000). Involvement of mitochondria in oxidative stress-induced cell death in mouse zygotes. *Biology of reproduction*, *62*(6), 1745-1753.

Liu, N., Wu, Y. G., Lan, G. C., Sui, H. S., Ge, L., Wang, J. Z., Liu, Y., Qiao, T. W., & Tan, J. H. (2009b). Pyruvate prevents aging of mouse oocytes. *Reproduction*, *138*(2), 223.

Lord, T., & Aitken, R. J. (2013). Oxidative stress and ageing of the post-ovulatory oocyte. *Reproduction*, *146*(6), R217-R227.

Lord, T., Nixon, B., Jones, K. T., & Aitken, R. J. (2013). Melatonin prevents postovulatory oocyte aging in the mouse and extends the window for optimal fertilization in vitro. *Biology of reproduction*, *88*(3), 67-1.

Lubzens, E., Young, G., Bobe, J., & Cerdà, J. (2010). Oogenesis in teleosts: how fish eggs are formed. *General and comparative endocrinology*, *165*(3), 367-389.

Maneechot, N., Yano, C. F., Bertollo, L. A. C., Getlekha, N., Molina, W. F., Ditcharoen, S., ... & de Bello Cioffi, M. (2016). Genomic organization of repetitive DNAs highlights chromosomal evolution in the genus Clarias (Clariidae, Siluriformes). *Molecular cytogenetics*, *9*(1), 1-10.

Miao, Y. L., Liu, X. Y., Qiao, T. W., Miao, D. Q., Luo, M. J., & Tan, J. H. (2005). Cumulus cells accelerate aging of mouse oocytes. *Biology of reproduction*, *73*(5), 1025-1031.

Miquel, J., Economos, A. C., Fleming, J., & Johnson Jr, J. E. (1980). Mitochondrial role in cell aging. *Experimental gerontology*, *15*(6), 575-591.

Mok, H. J., Shin, H., Lee, J. W., Lee, G. K., Suh, C. S., Kim, K. P., & Lim, H. J. (2016). Age-associated lipidome changes in metaphase II mouse oocytes. *PloS one*, *11*(2), e0148577.

Nasr-Esfahani, M. M., & Johnson, M. H. (1991). The origin of reactive oxygen species in mouse embryos cultured in vitro. *Development*, *113*(2), 551-560.

Oliver, C. N., Ahn, B. W., Moerman, E. J., Goldstein, S., & Stadtman, E. R. (1987). Age-related changes in oxidized proteins. *Journal of Biological Chemistry*, *262*(12), 5488-5491.

Ono, T., Mizutani, E., Li, C., Yamagata, K., & Wakayama, T. (2011). Offspring from intracytoplasmic sperm injection of aged mouse oocytes treated with caffeine or MG132. *Genesis*, *49*(6), 460-471.

Perez, G. I., Jurisicova, A., Matikainen, T., Moriyama, T., Kim, M. R., Takai, Y., Pru, J. K., Kolesnick, R. N., & Tilly, J. L. (2005). A central role for ceramide in the age-related acceleration of apoptosis in the female germline. *The FASEB journal*, *19*(7), 1-23.

Perez, G. I., Trbovich, A. M., Gosden, R. G., & Tilly, J. L. (2000). Mitochondria and the death of oocytes. *Nature*, *403*(6769), 500-501.

Petri, T., Dankert, D., Demond, H., Wennemuth, G., Horsthemke, B., & Grümmer, R. (2020). In vitro postovulatory oocyte aging affects H3K9 trimethylation in two-cell embryos after IVF. *Annals of Anatomy-Anatomischer Anzeiger*, *227*, 151424.

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free radicals: properties, sources, targets, and their implication in various diseases. *Indian journal of clinical biochemistry*, *30*(1), 11-26.

Rime, H., Guitton, N., Pineau, C., Bonnet, E., Bobe, J., & Jalabert, B. (2004). Post-ovulatory ageing and egg quality: a proteomic analysis of rainbow trout coelomic fluid. *Reproductive Biology and Endocrinology*, *2*(1), 1-10.

Romanova, E. M., Lyubomirova, V. N., Romanov, V. V., Mukhitova, M. E., & Shlenkina, T. M. (2018). Seasonal studies of caviar production and the growth rate of the African catfish (Clarias gariepinus, Burchell, 1822). *The Egyptian Journal of Aquatic Research*, 44(4), 315-319.

Samarin, A. M., Amiri, B. M., Soltani, M., Nazari, R. M., Kamali, A., & Naghavi, M. R. (2011). Effects of storage duration and storage temperature on viability of stored ova of kutum (Rutilus frisii kutum) in ovarian fluid. *African Journal of Biotechnology*, *10*(57), 12309-12314.

Samarin, A. M., Blecha, M., Uzhytchak, M., Bytyutskyy, D., Zarski, D., Flajshans, M., & Policar, T. (2016). Post-ovulatory and post-stripping oocyte ageing in northern pike, Esox lucius (Linnaeus, 1758), and its effect on egg viability rates and the occurrence of larval malformations and ploidy anomalies. *Aquaculture*, *450*, 431-438.

Samarin, A. M., Policar, T., & Lahnsteiner, F. (2015). Fish oocyte ageing and its effect on egg quality. *Reviews in Fisheries Science & Aquaculture*, *23*(3), 302-314.

Samarin, A. M., Samarin, A. M., & Policar, T. (2019a). Cellular and molecular changes associated with fish oocyte ageing. *Reviews in Aquaculture*, *11*(3), 619-630.

Samarin, A. M., Samarin, A. M., Østbye, T. K. K., Ruyter, B., Sampels, S., Burkina, V., Blecha, M., & Policar, T. (2019b). The possible involvement of oxidative stress in the oocyte ageing process in goldfish Carassius auratus (Linnaeus, 1758). *Scientific reports*, *9*(1), 1-9.

Samarin, A. M., Samarin, A.M., Østbye, T. K. K., Ruyter, B., Sampels, S., Burkina, V., Blecha, M., Gela, D., & Policar, T. (2019c). Alteration of mRNA abundance, oxidation products and antioxidant enzyme activities during oocyte ageing in common carp Cyprinus carpio. *PLoS One*, *14*(2), e0212694.

Samarin, A. M., Sampels, S., Krzyskow, A., Burkina, V., Kristan, J., Gela, D., Flajshans M., Policar T., & Samarin, A. M. (2018a). Egg oxidation status, antioxidant enzyme activities, lipid classes, fatty acid composition profile and embryo survival rates during in vitro oocyte ageing in tench Tinca tinca (Linnaeus, 1758). *Aquaculture Research*, *49*(6), 2305-2316.

Samarin, A. M., Sampels, S., Policar, T., Rodina, M., Hematyar, N., & Samarin, A. M. (2018b). mRNA abundance changes during in vitro oocyte ageing in African catfish Clarias gariepinus (Burchell, 1822). Aquaculture Research, 49(2), 1037-1045.

Samarin, A.M., (2018). Alteration of mRNA abundance, oxidation products and antioxidant enzyme activities associated with fish oocyte ageing, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, Vodňany, Czech Republic.

Simsek-Duran, F., Li, F., Ford, W., Swanson, R. J., Jones Jr, H. W., & Castora, F. J. (2013). Age-associated metabolic and morphologic changes in mitochondria of individual mouse and hamster oocytes. *PloS one*, *8*(5), e64955.

Suzuki, Y. J., Carini, M., & Butterfield, D. A. (2010). Protein carbonylation. *Antioxidants & redox signaling*, *12*(3), 323-325.

Tafani, M., Sansone, L., Limana, F., Arcangeli, T., De Santis, E., Polese, M., Fini, M., & Russo, M. A. (2016). The interplay of reactive oxygen species, hypoxia, inflammation, and sirtuins in cancer initiation and progression. *Oxidative medicine and cellular longevity*, *2016*, 3907147.

Takahashi, T., Igarashi, H., Amita, M., Hara, S., Matsuo, K., & Kurachi, H. (2013). Molecular mechanism of poor embryo development in postovulatory aged oocytes: mini review. *Journal of Obstetrics and Gynaecology Research*, *39*(10), 1431-1439.

Takahashi, T., Igarashi, H., Kawagoe, J., Amita, M., Hara, S., & Kurachi, H. (2009). Poor embryo development in mouse oocytes aged in vitro is associated with impaired calcium homeostasis. *Biology of reproduction*, *80*(3), 493-502.

Takahashi, T., Takahashi, E., Igarashi, H., Tezuka, N., & Kurachi, H. (2003). Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. *Molecular Reproduction and Development: Incorporating Gamete Research*, *66*(2), 143-152.

Tamura, H., Takasaki, A., Miwa, I., Taniguchi, K., Maekawa, R., Asada, H., Taketani, T., Matsuoka A., Yamagata, Y., Shimamura, K., Morioka, H., Ishikawa, H., Reiter, R., J., & Sugino, N. (2008). Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *Journal of pineal research*, *44*(3), 280-287.

Tarín, J. J. (1995). Cell cycle: Aetiology of age-associated aneuploidy: a mechanism based on the ‘free radical theory of ageing’. *Human Reproduction*, *10*(6), 1563-1565.

Tarín, J. J., Pérez-Albalá, S., & Cano, A. (2000). Consequences on offspring of abnormal function in ageing gametes. *Human Reproduction Update*, *6*(6), 532-549.

Tarín, J. J., Pérez-Albalá, S., & Cano, A. (2002). Oral antioxidants counteract the negative effects of female aging on oocyte quantity and quality in the mouse. *Molecular reproduction and development*, *61*(3), 385-397.

Tarín, J. J., Ten, J., Vendrell, F. J., & Cano, A. (1998). Dithiothreitol prevents age-associated decrease in oocyte/conceptus viability in vitro. *Human Reproduction*, 13(2), 381-386.

Tata, J. R. (1986). Coordinated assembly of the developing egg. *Bioessays*, *4*(5), 197-201.

Tesfahun, A. (2018). Feeding biology of the African catfish Clarias gariepinus (Burchell) in some of Ethiopian Lakes: A review. *International Journal of Fauna and Biological Studies*, *5*(1), 19-23.

Teugels, G. G., & Gourene, G. (1998). Biodiversity and aquaculture of African catfishes (Teleostei, Siluroidei): an overview. In Agnèse, J. F., *Genetics and aquaculture in Africa* (229-240). Paris, France: Editions de l’Orstom.

Vitule, J. R., Umbria, S. C., & Aranha, J. M. R. (2006). Introduction of the African catfish Clarias gariepinus (Burchell, 1822) into Southern Brazil. *Biological Invasions*, *8*(4), 677-681.

Wang, L., Tang, J., Wang, L., Tan, F., Song, H., Zhou, J., & Li, F. (2021). Oxidative stress in oocyte aging and female reproduction. *Journal of Cellular Physiology*, 1-18.

Wesson, D. E., & Elliott, S. J. (1995). The H2O2-generating enzyme, xanthine oxidase, decreases luminal Ca^{2+} content of the IP3-sensitive Ca^{2+} store in vascular endothelial cells. *Microcirculation*, *2*(2), 195-203.

Wu, Y., Wang, X. L., Liu, J. H., Bao, Z. J., Tang, D. W., Wu, Y., & Zeng, S. M. (2011). BIMEL-mediated apoptosis in cumulus cells contributes to degenerative changes in aged porcine oocytes via a paracrine action. *Theriogenology*, *76*(8), 1487-1495.

Zeb, A., & Ullah, F. (2016). A simple spectrophotometric method for the determination of thiobarbituric acid reactive substances in fried fast foods. *Journal of analytical methods in chemistry*, *2016*.

Zhang, N., Wakai, T., & Fissore, R. A. (2011). Caffeine alleviates the deterioration of Ca^{2+} release mechanisms and fragmentation of in vitro-aged mouse eggs. *Molecular reproduction and development*, *78*(9), 684-701.