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Alteration of mRNA abundance, oxidation products and antioxidant enzyme activities associated with fish oocyte ageing

Změny v množství mRNA, v aktivitě oxidativní produktů a antioxidačních enzymů v závislosti na stárnutí rybích oocytů

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Czech Republic, Vodňany, 2018

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Oxidative stress and fish oocyte ageing

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1.1. OXIDATIVE STRESS AND FISH OOCYTE AGEING

Varying egg quality and a loss of oocyte viability after ovulation are the limiting factors in the reproduction and mass production of several fish species (Furuita et al. 2003; Rizzo et al. 2003). In fish farming, egg quality is defined by the ability to be fertilized and subsequently develop into a normal embryo and is dependent on several intrinsic and environmental factors (Bobe & Labbe 2010). Oocyte ageing has been identified as the most important factor affecting egg quality in several fish species after ovulation (*e.g.,* Lam et al. 1978; Rime et al. 2004). In fish, ovulated oocytes are arrested at the metaphase II stage of meiotic division. These oocytes then await fertilization by the spermatozoon. Delayed fertilization within this time results in the occurrence of deleterious processes in the oocyte quality, known as post-ovulatory oocyte ageing. Ageing of the ovulated oocytes may occur either *in vivo* or *in vitro* conditions. If the ovulated eggs age inside the fish body due to finding the proper spawning habitat or due to environmental conditions caused by humans, such as dam building that delays fish spawning (Gaudemar and Beall, 1998), oocytes will undergo ageing *in vivo* conditions. Once the eggs are not fertilized after stripping, the eggs will be aged *in vitro* conditions. In the artificial propagation of cultured fish, the females are examined for ovulation from time to time to avoid the ageing of ovulated eggs.

The optimum time for fertilization after ovulation and/or stripping may vary from a few minutes to a few weeks depending on the fish species and water temperature (Samarin et al. 2015a). Oocyte ageing in fish is associated with a limited fertilization rate (Lahnsteiner et al. 2001; Samarin et al. 2011), increased larval malformation (Aegerter & Jalabert 2004; Rime et al. 2004; Bonnet et al. 2007a) and increased ploidy anomalies (Flajshans et al. 2007; Aegerter & Jalabert 2004). The effect of fish oocyte ageing on the morphological, physiological, biochemical and histological characteristics of resulting eggs is reviewed by Samarin et al. (2015a). In other vertebrates, the oocyte ageing process has been shown to be associated with polyspermy, poor embryo development (Chian et al. 1992) and offspring abnormalities arising from epigenetic changes (e.g., Tarin et al. 2002; Liang et al. 2011; Liang et al. 2008). Embryo quality and the later life of the offspring are highly dependent on oocyte integrity, as the oocyte contains important information to orchestrate embryogenesis (Minami et al. 2007) and to remodel the parental genomes (Yoshida et al. 2007). Other deleterious effects related to oocyte ageing include premature hardening of the zona radiata and cortical granule exocytosis (Xu et al. 1997), a decrease in critical cell cycle factors (Kikuchi et al. 2002), and meiotic spindle and chromosomal abnormalities (Wakayama et al. 2004). Apoptotic cell death is the end point of the oocyte ageing process.

Until now, there has been only a poor understanding about the processes and underlying mechanisms of oocyte ageing in fish as well as in other vertebrates. Few studies have analysed the transcriptome changes and egg quality associated with oocyte ageing in fish (Aegerter, Jalabert & Bobe, 2005; Bonnet, Fostier & Bobe, 2007b; Ma et al. 2015; Mommens et al. 2010). Different quantities of mRNAs between over-ripened and freshly ovulated rainbow trout *Oncorhynchus mykiss* eggs have been reported (Aegerter et al. 2004; Aegerter et al. 2005; Bonnet et al. 2007b). In the Atlantic halibut, *Hippoglossus hippoglossus*, poor hatching success was correlated with the low transcript levels of specific genes (Mommens et al., 2010). In rainbow trout eggs, ova ageing results in the downregulated expression of specific microRNAs and their target genes, which are mainly involved in cell death and signal transduction, stress response and DNA damage, RNA degradation, and energy and transcription regulation (Ma et al. 2015). Age-associated alterations in gene expression during mice and human oocyte ageing have also identified candidate mRNAs and proteins involved in the spindle assembly checkpoint and regulation and control of the cell cycle, particularly those

related to mitochondrial function and energy pathways (Hamatani et al. 2004; Steuerwald, Bermudez, Wells, Munne & Cohen, 2007). However, the specific molecular functions that determine egg quality associated with oocyte ageing in fish and in other vertebrates remain to be elucidated.

Studies on other vertebrates have proposed the involvement of oxidative stress as the initiating factor in the progress of oocyte ageing (*e.g.,* Tarin et al. 2000; Takahashi et al. 2003; Lord et al. 2013). These studies report that oxidative stress can, in turn, trigger many cascades affecting oocyte quality, such as mitochondrial dysfunction, DNA damage, perturbed Ca^{2+} homeostasis and lipid peroxidation. In general, ageing is associated with increases in the levels of endogenous Reactive oxygen species (ROS) and decreases in antioxidant defences, leading to a wide range of oxidative damage in cell structures, including lipid peroxidation of membranes, enzyme inactivation, protein oxidation, and DNA damage (Dean et al. 1993; Headlam and Davies 2004; Orrenius et al. 2007). Alteration of the lipidome, associated with oocyte ageing, was evaluated in a mouse model (Mok et al. 2016). The latter study reported that several phospholipid classes significantly decreased in aged oocytes, which suggests the involvement of oxidative stress in lipid plasma membrane composition and, as a result, unfavourable outcomes of oocyte ageing. Therefore, the levels of fatty acids and lipid class composition were measured during prolonged *in vitro* oocyte ageing. Enzymatic antioxidant systems can scavenge ROS and, therefore, decrease the effect of oxidative stress. The role of oxidative stress in the ageing of several types of cells and tissues has been implicated, but very little is known about how oocyte antioxidant defences change during oocyte ageing. Declines in critical cell cycle factors (Kikuchi et al. 2002) and impaired mitochondrial function (*e.g.,* Tarin et al. 2002; Hamatani et al. 2004) are shown be related to the deleterious effects of oocyte ageing.

The present study examined some cellular and molecular changes associated with fish oocyte ageing, focusing on the possible role of oxidative stress in the progress of an oocyte time dependent over-ripening process. The evaluation was done at the levels of transcriptome and antioxidant enzyme assays, lipid and protein oxidation status, fatty acid and lipid class composition, as well as the egg phenotype and functional changes during the oocyte ageing. The genes involved in oxidative damage and stress response, mitochondrial function, with roles in fertilization, embryo development, transcriptional regulation and cell cycling, as well as the ones related to the apoptosis were screened for their mRNA abundance during the egg over-ripening. To study how maternal transcripts influence egg quality, these parameters were correlated to embryo developmental stages at varying post-ovulatory aged times. In addition, we chose to investigate a possible alteration in the oxidation status of oocytes during post-ovulatory ageing by measuring TBARs as the marker of lipid oxidation and carbonyls, which show the extension of protein oxidation. In addition, the role of oxidative stress in the progress of fish oocyte ageing was assessed by an evaluation of the oxidation status of the oocytes during post-ovulatory ageing. We investigated the activity of the following antioxidant enzymes, CAT (Catalase), SOD (Superoxide dismutase), GPX (Glutathione peroxidase) and GR (Glutathione reductase), during fish oocyte ageing. The combination of the abovementioned parameters will give a broad picture and understanding of the ongoing mechanisms in the oocyte ageing process. Identifying molecular mechanisms involved in the decline of oocyte quality with the progress of oocyte ageing could have important implications for aspects of basic research and practically applications for aquaculture purposes to prevent or delay the oocyte ageing.

THESIS AIM AND SPECIFIC OBJECTIVES

The aim of the current thesis is to investigate the role of oxidative stress, mitochondrial dysfunction, cell cycling and apoptotic related indices on the progress of fish oocyte ageing. An investigation on oocytes of different ages was performed and analysed at the level of the transcriptome. Lipid oxidation, protein oxidation and antioxidant enzyme assays were also performed.

Specific objectives:

1. Examine the mRNA abundance of the selected genes associated with oxidative injury and stress response during oocyte ageing;
2. Examine the mRNA abundance of the selected genes associated with mitochondrial dysfunction during oocyte ageing;
3. Examine the mRNA abundance of the selected genes associated with the cell cycling during oocyte ageing;
4. Examine the mRNA abundance of the selected genes associated with apoptosis during oocyte ageing;
5. Examine the levels of fatty acids, the lipid class composition, and the markers of oxidative damage on lipids and proteins during prolonged *in vitro* oocyte ageing in tench (*Tinca tinca*);
6. Examine the probable alterations in the activity of the egg antioxidant enzymes during oocyte ageing in tench, goldfish *(Carassius auratus*) and common carp (*Cyprinus carpio*);
7. Examine the egg phenotype and functional changes.

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: 59– 71.

Aegerter S, Jalabert B, Bobe J (2005) Large scale real-time PCR analysis of mRNA abundance in rainbow trout eggs in relationship with egg quality and post-ovulatory ageing. *Molecular Reproduction and Development* 72: 377–385.

Aegerter, S., Jalabert, B. and Bobe, J. (2004) MessengerRNAstockpile of cyclin B, insulin-like growth factor I, insulin-like growth factor II, insulinlike growth factor receptor Ib, and p53 in the rainbow trout oocyte in relation with developmental competence. *Mol Reprod Dev* 67, 127–135.

Bobe J, Labbe C (2010) Egg and sperm quality in fish. *General and Comparative Endocrinology* 165: 535–548.

Bonnet E, Fostier A, Bobe J (2007a) Characterization of rainbow trout egg quality: A case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations. *Theriogenology* 67: 786–794.

Bonnet E, Fostier A, Bobe J (2007b) Microarray-based analysis of fish egg quality after natural or controlled ovulation. *BMC Genomics* 8: 55.

Chian RC, Nakahara H, Niwa K, Funahashi H (1992) Fertilization and early cleavage in vitro of ageing bovine oocytes after maturation in culture. *Theriogenology* 37: 665–672.

Dean, R.T., Gieseg, S. & Davies, M.J. (1993) Reactive species and their accumulation on radical-damaged proteins. *Trends in Biochemical Sciences* 18, 437–441.

Flajshans M, Kohlmann K, Rab P (2007) Autotriploid tench *Tinca tinca (L.*) larvae obtained by fertilization of eggs previously subjected to postovulatory ageing *in vitro* and *in vivo*. *Journal of Fish Biology* 71: 868–876.

Furuita, H., H. Ohta, T. Unuma, H. Tanaka, H. Kagawa, N. Suzuki, and T. Yamamoto. Biochemical composition of eggs in relation to egg quality in the Japanese eel Anguilla japonica. Fish Physiol. Biochem., 29: 37–46 (2003).

Gaudemar, B. DE. and E. Beall. Effects of overripening on spawning behaviour and reproductive success of Atlantic salmon females spawning in a controlled flow channel. J. Fish biol., 53: 434–446 (1998).

Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, et al (2004) Age-associated alteration of gene expression patterns in mouse oocytes. *Human Molecular Genetics* 13: 2263–2278.

Headlam, H.A. & Davies, M.J. (2004) Markers of protein oxidation: different oxidants give rise to variable yields of bound and released carbonyl products. *Free Radical Biology and Medi*cine 36, 1175–1184.

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 and Stem Cells* 4: 211–222.

Lahnsteiner F, Urbanyi B, Horvath A, Weismann T (2001) Biomarkers for egg quality determination in cyprinid fish. *Aquaculture* 195: 331–352.

Lam TJ, Nagahama Y, Chan K, Hoar WS (1978) Overripen eggs and post-ovulatory corpora lutea in the three spined stickleback. *Canadian Journal of Zoology* 56: 2029–2036.

Liang XW, Ge ZI, Wei L, Guo L, Han ZM, Schatten H, Sun QY (2011) The effects of postovulatory aging of mouse oocytes on methylation and expression of imprinted genes at mid-term gestation. *Molecular Human Reproduction* 17: 562–567.

Liang XW, Zhu JQ, Miao YL, Liu JH, Wei L, Lu SS, et al (2008) Loss of methylation imprint of Snrpn in postovulatory aging mouse oocyte. *Biochemical and Biophysical Research Communications* 371: 16–21.

Lord T, Nixon B, Jones KT, Aitken RJ (2013) Melatonin prevents postovulatory oocyte aging in the mouse and extends the window for optimal fertilization *in vitro*. *Biology of Reproduction* 88: 1–9.

Ma H, Weber GM, Hostuttler MA, Wei H, Wang L, Yao J (2015) MicroRNA expression profiles from eggs of different qualities associated with post-ovulatory ageing in rainbow trout (*Oncorhynchus mykiss*). *BMC Genomics* 16: 201.

Minami N, Suzuki T, Tsukamoto S (2007) Zygotic gene activation and maternal factors in mammals. *The Journal of Reproduction and Development* 53: 707–715.

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, e0148577.

Mommens, M., Fernandes, J.M., Bizuayehu, T.T., Bolla, S.L., Johnston, I.A. and Babiak, I. (2010) Maternal gene expression in Atlantic halibut (*Hippoglossus hippoglossus* L.) and its relation to egg quality. *BMC Res. Notes* 3, 138.

Orrenius, S., Gogvadze, V. & Zhivotovsky, B. (2007) Mitochondrial oxidative stress: implications for cell death. *Annu Rev Pharmacol Toxicol* 47, 143–183.

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: 26–36.

Rizzo, E., H. P. Godinho, and Y. Sato. Short-term storage of oocytes from the neotropical teleost fish Prochilodus marggravii. Theriogenology, 60: 1059–1070 (2003).

Samarin AM, Mojazi Amiri B, Soltani M, Nazari RM, Kamali A, Naghavi MR (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: 12309–12314.

Samarin AM, Policar T, Lahnsteiner F (2015a) Fish Oocyte Ageing and its Effect on Egg Quality. *Reviews in Fisheries Science & Aquaculture* 23: 302–314.

Steuerwald NM, Bermudez MG, Wells D, et al., (2007) Maternal age-related differential global expression profiles observed in human oocytes. Reprod Biomed Online 14: 700-708.

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* 66: 143–152.

Tarin JJ, Perez-Albala S, Perez-Hoyos P, Cano A (2002) Postovulatory aging of oocytes decreases reproductive fitness and longevity of offspring. *Biology of Reproduction* 66: 495–499.

Tarin, J.J., Perez-Albala, S. and Cano, A. (2000) Consequences on offspring of abnormal function in ageing gametes. *Human Reproduction Update* 6, 532–549.

Wakayama S, Thuan NV, Kishigami S, Ohta H, Mizutani E, Hikichi T, et al (2004) Production of offspring from one-day-old oocytes stored at room temperature. *Journal of Reproduction and Development* 50: 627–637.

Xu Z, Abbott A, Kopf GS, Schultz RM, Ducibella T (1997) Spontaneous activation of ovulated mouse eggs: time-dependent effects on M-phase exit, cortical granule exocytosis, maternal messenger ribonucleic acid recruitment, and inositol 1,4,5-triphosphate sensitivity. *Biology of Reproduction* 57: 743–750.

Yoshida N, Brahmajisyula M, Shoji S, Amanai M, Perry AC (2007) Epigenetic discrimination by mouse metaphase II oocytes mediated asymmetric chromatin remodelling independently of meiotic exit. *Developmental Biology* 301: 464–477.

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Cellular and molecular changes associated with fish oocyte ageing

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Abstract

Post-ovulatory oocyte ageing in fish is associated with limited fertilization rate, poor embryo development, larval malformation, ploidy anomalies and various other offspring abnormalities. Many studies have focused on morphological, physiological, biochemical and histological changes that occur during oocyte ageing. However, little is known about molecular changes associated with this process. This review covers the cellular and molecular characteristics of ageing oocytes with special focus on age-associated changes affected by oxidative damage. The contribution of oxidative stress and mitochondrial dysfunction, as well as new insights into oocyte ageing in higher vertebrates, is discussed to provide direction for future studies. Investigating the genomics and proteomics of oocyte ageing, including underlying molecular mechanisms, could contribute valuable information on determinants of egg quality. Such knowledge can be translated into practical applications in aquaculture, such as supplementation of oocyte culture media with antioxidants and other agents as a means to inhibit or delay development of the aged oocyte phenotype. Identification of cellular and molecular markers associated with the oocyte ageing process will benefit such developments.

Key words: cellular changes, fertilizing ability, fish oocyte ageing, molecular changes, molecular markers, oxidative stress.

Introduction

Controlling egg quality is of great interest and importance in aquaculture and other animal breeding programmes. In fish farming, egg quality is defined by the ability to be fertilized and subsequently develop into normal embryos and is dependent on several intrinsic and environmental factors (Bobe & Labbe 2010). Oocyte ageing after ovulation has been identified as the most important factor affecting egg quality in several fish species (e.g. Lam *et al.* 1978; Rime *et al.* 2004). In fish, ageing of the unfertilized metaphase II stage oocyte occurs over time after ovulation. Delayed spawning in nature, delayed egg collection in captivity and delayed fertilization after egg stripping all lead to excessive oocyte ageing sometimes referred to as 'over-ripening'. The optimum time of fertilization after ovulation and/or stripping may vary from a few minutes to a few weeks depending on the fish species and water temperature (for details, see Samarin *et al.* 2015b). Post-ovulatory and post-stripping oocyte ageing in fish is associated with a limited fertilization rate (e.g. Lahnsteiner *et al.* 2001; Samarin *et al.* 2011), increased larval malformation (e.g. Aegerter & Jalabert 2004; Rime *et al.* 2004; Bonnet *et al.* 2007a) and increased ploidy anomalies (e.g. Aegerter & Jalabert 2004; Flajshans *et al.* 2007). In higher vertebrates, the oocyte ageing process has been shown to be associated with polyspermy, poor embryo development (Chian *et al.* 1992) and offspring abnormalities arising from epigenetic changes (e.g. Tarin *et al.* 2002; Liang *et al.* 2008, 2011). Embryo quality and later life of the offspring are highly dependent on oocyte integrity as the oocyte contains important information to orchestrate embryogenesis (Minami *et al.* 2007) and to remodel the parental genomes (Yoshida *et al.* 2007). Other deleterious effects related to oocyte ageing include premature hardening of the zona radiata and cortical granule exocytosis (Xu *et al.* 1997), decrease in critical cell cycle factors (Kikuchi *et al.* 2002) and meiotic spindle and chromosomal abnormalities (Wakayama *et al.* 2004). Apoptotic cell death is the end point of the oocyte ageing process.

The effect of fish oocyte ageing on the morphological, physiological, biochemical and histological characteristics of

resulting eggs was recently reviewed by Samarin *et al.* (2015b). However, the molecular changes that occur during fish oocyte ageing have not previously been reviewed. The precise molecular mechanisms underlying deleterious effects of oocyte ageing are unknown, but studies of higher vertebrates have suggested the involvement of oxidative stress (e.g. Tarin *et al.* 2000; Takahashi *et al.* 2003; Lord *et al.* 2013), decline in critical cell cycle factors (Kikuchi *et al.* 2002) and impaired mitochondrial function (e.g. Tarin *et al.* 2002; Hamatani *et al.* 2004). Fish are good model animals to evaluate oocyte ageing because they display a vast diversity of reproductive modes, and most species produce a high number of oocytes compared with other animals. Furthermore, as the demand for assisted reproduction technology is increasing, studies of the process of oocyte ageing in fish might be beneficial for higher vertebrates as well.

In the present article, the cellular and molecular changes during fish oocyte ageing are reviewed with a particular focus on genes involved in oxidative stress and mitochondrial dysfunction, the cell cycle, DNA and chromosome stability, apoptosis, fertilization, embryo development and transcriptional regulation. The possibility of delaying or inhibiting oocyte ageing by supplementing the oocyte culture media with antioxidants and other agents is also discussed.

Cellular changes during the progress of oocyte ageing

It has not yet been clearly determined whether the oocyte ageing process starts via action of an initiating factor that triggers a series of events finally leading to loss of fertility or whether different cellular and molecular events are initiated separately and then come together to complete the process of over-ripening. Most authors believe that the onset of ageing in oocytes is associated with an increase in reactive oxygen species (ROS) (Fig. 1) (e.g. Tarin *et al.* 2000; Takahashi *et al.* 2003; Lord & Aitken 2013; Lord *et al.* 2013). The main consequences of oxidative stress in the oocytes are ROS-induced mitochondrial dysfunction, lipid alterations and DNA fragmentation followed by ATP depletion and apoptosis. Increased oxidative stress in oocytes may be associated with reduced fertilization success, diminished embryo quality and increased abnormalities observed in the later life of the offspring (Tarin *et al.* 2000; Lord & Aitken 2013). In mice, even brief oxidative stress in unfertilized oocytes can lead to significant negative impacts on embryo development (Takahashi *et al.* 2003). In the same species, Hamatani *et al.* (2004) also reported that the duration of increased oxidative stress contributes to maternal oocyte ageing. Takahashi *et al.* (2011) reviewed two categories of oocyte ageing caused by ovarian ageing or post-ovulatory ageing. The authors concluded that both categories of oocyte ageing have similar phenotypes of reproductive failure. However, the mechanisms for the impairment in oocyte quality are not necessarily equivalent. In spite of the major differences between maternal and post-ovulatory oocyte ageing in oocyte stage, follicular environment and ageing interval, certain commonalities appear to exist between follicle-enclosed oocytes undergoing ageing

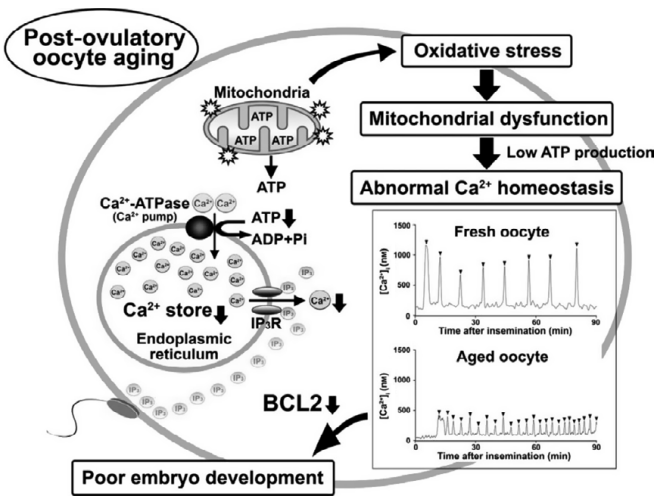

Figure 1 Schematic of the mechanism of poor embryo development in post-ovulatory oocyte ageing (obtained from Takahashi *et al.* 2013).

in situ, which may occur over many years, and oocytes undergoing post-ovulatory ageing for short periods *in vivo* or *in vitro*.

Hypoxia, and rebound from hypoxia, is intimately linked to ROS production and to apoptosis (Tafani *et al.* 2016). Although the precise mechanism through which hypoxia increases ROS is not yet well known, it seems that ROS production is due to the effects of hypoxia on the mitochondria electron transport chain (Tafani *et al.* 2016). During ageing, oocytes experience an increase in oxidative stress due to their cellular metabolism, the culture environment and the lack of protective antioxidant mechanisms that are naturally present in their intrafollicular habitat. Diminished oxygen concentrations stimulate the expression of hypoxia-inducible factor (HIF), a transcription factor that up- and downregulates many target genes, including modulation of apoptotic genes, creation of pro-inflammatory states and alteration of the redox status of the cell (Combelles *et al.* 2009).

The scavenging of ROS is a promising therapeutic target for hypoxia injury. The general superoxide dismutase (SOD)-mimetic drug, Tempol (4-Hydroxy-TEMPO), which efficiently neutralizes ROS, reduces the hypoxia-induced oxidant stress and apoptosis by scavenging free radicals and modulating the expression of apoptosis-related proteins (Jing *et al.* 2017). Hypoxia upregulated *bax* and *caspase-3* expression and downregulated *bcl2* expression. Pretreatment with Tempol downregulated the level of mRNA expressions of *bax* and *caspase-3* and upregulated the expression of *bcl2*. In addition, hypoxia exposure significantly upregulated the protein expression of Bax/Bcl-2 ratio and the expression of caspase-3 (Jing *et al.* 2017) (Fig. 2).

ROS-induced mitochondrial dysfunction and disturbance in intracellular Ca^{2+} regulation in aged oocytes

Studies on higher vertebrates have indicated that elevated ROS levels in aged oocytes significantly disturb mitochondrial function (Takahashi *et al.* 2003). Mitochondrial ATP production is necessary for many processes in oocytes, including Ca^{2+} homeostasis. Hamatani *et al.* (2004) observed a decrease in transcript levels of ATP-related genes in mouse oocytes during maternal ageing. An important consequence of mitochondrial dysfunction is impaired intracellular Ca^{2+} regulation observed in the aged oocytes following fertilization (Takahashi *et al.* 2003). The oscillation of Ca^{2+} levels following fertilization exhibits a higher frequency but lower amplitude in more aged oocytes than in fresh oocytes (e.g. Takahashi *et al.* 2003, 2009). Reuptake of Ca^{2+} by Ca^{2+} ATPase in the endoplasmic reticulum (ER) is impaired in aged oocytes (Igarashi *et al.* 1997), and Ca^{2+} release from 1,4,5-triphosphate (InsP3)-sensitive Ca^{2+} stores is diminished in aged oocytes due to depletion of the ER Ca^{2+} stores (Wesson & Elliott 1995; Takahashi *et al.* 2000). Exposure of fresh oocytes to oxidative stress changes the Ca^{2+} oscillation pattern towards one similar to that seen in aged oocytes (Igarashi *et al.* 1997; Takahashi *et al.* 2000, 2003) and also results in poor embryo development (Takahashi *et al.* 2009). In addition, ROS can directly affect the Ca^{2+} binding protein calmodulin. Impaired Ca^{2+} homeostasis in aged oocytes can subsequently contribute to apoptosis (Takahashi *et al.* 2013).

In aged oocytes, mitochondrial membrane potential is decreased and swelling of the mitochondrial matrix occurs (Wilding *et al.* 2001). It is well known that maternal ageing involves a decline in the number and function of mitochondria in oocytes (Simsek-Duran *et al.* 2013). However, it has been recently proposed that mitochondrial damage in post-ovulatory-aged oocytes is even much more severe than that observed in maternally aged oocytes (Yamada-Fukunaga *et al.* 2013). Lord *et al.* (2013) suggested that increased oxidative stress can be considered as an early marker of oocyte ageing before the activation of caspase-3 and also before the appearance of the morphological features of oocyte ageing and apoptosis. Mitochondria are the main targets of ROS-induced oxidative stress, and post-ovulatory and maternal ageing of oocytes both involve altered redox state and mitochondrial function defects (Takahashi *et al.* 2009).

ROS-induced lipid oxidation in aged oocytes

It has been reported in a mouse model that lipid peroxidation in the membranes of oocytes aged *in vivo* is higher than seen in fresh oocytes (Takahashi *et al.* 2003). A reduction in membrane fluidity is observed following lipid peroxidation in the oocyte membrane that might cause decreased fusion of sperm and oolemma, resulting in a decreased fertilization rate in aged oocytes (Lord & Aitken 2013).

Levels of glutathione (GSH), which is considered a ROS scavenger, are decreased with prolonged latency of ovulation to fertilization (Boerjan & de Boer 1990). Exposure of oocytes to oxidative stress results in a decreased fertilization rate and apoptosis (Liu & Keefe 2000). In the human embryo, a positive correlation was reported between the concentration of hydrogen peroxide and apoptosis occurrence (Yang *et al.* 1998). Thus, the onset of oxidative stress in oocyte ageing is not unexpected considering the reported increase in ROS and lipid peroxidation levels and decline in the glutathione levels (Yoshida *et al.* 1993). In aged oocytes, the absence of follicular fluid, which is rich in antioxidants and plays a protective role against ROS damage, could also contribute to oxidative stress. Apoptotic cell death, which is the end

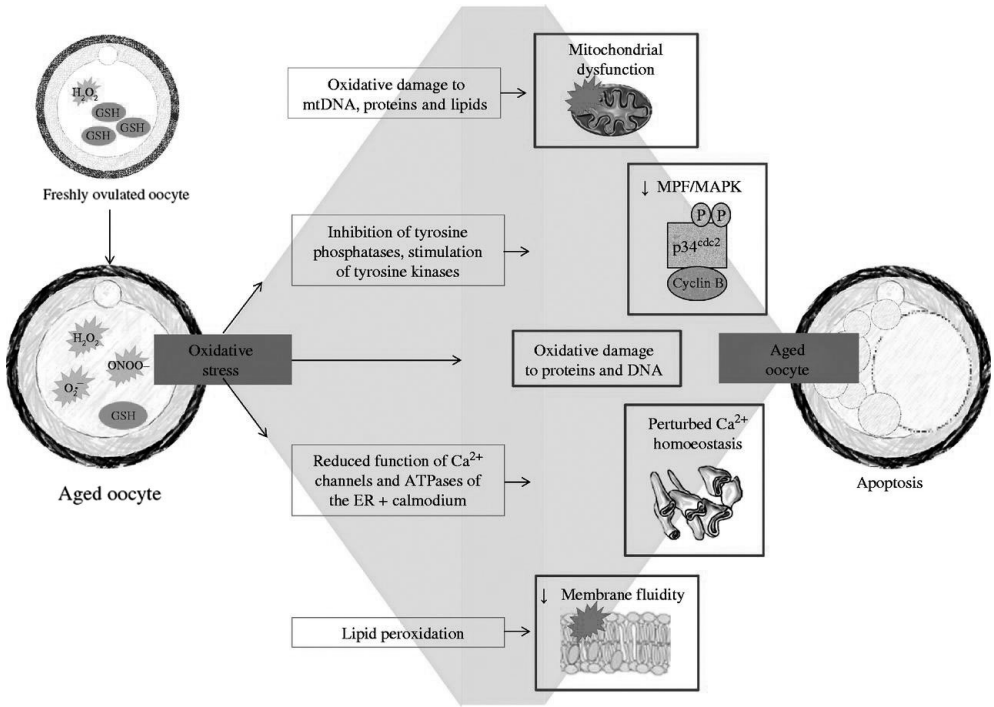

Figure 2 With increasing amounts of time following ovulation, MII stage oocytes experience an accumulation of reactive oxygen species and a depletion of glutathione resulting in the onset of oxidative stress. Oxidative stress has the potential to initiate the post-ovulatory ageing process via multiple pathways: (i) through oxidative damage to the mitochondria resulting in subsequent dysfunction, (ii) through inhibition of tyrosine phosphatases and stimulation of tyrosine kinases resulting in a decline in levels of maturation-promoting factor, (iii) by causing global damage to proteins and DNA, (iv) by reducing the function of Ca2C channels and ATPases in the endoplasmic reticulum as well as affecting the Ca2C signalling molecule calmodulin resulting in perturbed Ca2C homoeostasis and (v) through peroxidation of lipids in the plasma membrane, decreasing membrane fluidity and potentially affecting the oocytes' potential for fusion with the spermatozoon. The accumulation of these factors, potentially as a result of oxidative stress, results in the culmination of an 'aged' oocyte (Obtained from Lord & Aitken 2013).

point of the oocyte ageing process, might also be associated with increased oxidative stress and the altered redox status of oocytes (Ott *et al.* 2007).

Over-ripening of rainbow trout eggs was accompanied by an increase in free lipid and a decrease in lipid phosphorus, as measures of phospholipids, PhoLs (Craik & Harvey 1984). Additionally, the levels of esterified and non-esterified fatty acids are significantly decreased in over-ripened rainbow trout eggs (Lahnsteiner 2000). In Caspian brown trout, the level of triglycerides decreases 30 days post-ovulation (Bahrekazemi *et al.* 2010). More recently, Mok *et al.* (2016) also reported that several PhoL classes, including phosphatidic acid, phosphatidylinositol, phosphatidylserine and lysophosphatidylserine, significantly decreased both in H_2O_2-treated and aged oocytes.

ROS-induced chromosomal and DNA abnormalities in aged oocytes

Oocyte ageing is associated with changes in the meiotic spindle (Tarin *et al.* 2000). The meiotic spindle conveys the genetic information of the oocyte, contributing to proper embryo development through an accurate distribution of chromosomes to blastomeres. Furthermore, immunocytochemical studies have shown that human *in vitro* aged oocytes showed disrupted spindles, tetrapolar spindles and aberrant expression of the nuclear mitotic apparatus protein providing spindle stability in fresh oocytes, and also the microtubule kinesin motor protein EG5 (Hall *et al.* 2007). Additionally, oocyte ageing affects numerous changes in the chromosome alignment of mouse oocytes

(Wakayama *et al.* 2004). Advancing maternal age is a well-recognized factor influencing the occurrence the spindle abnormalities (Sanchez *et al.* 2017). However, this has not yet been studied with fish post-ovulatory oocyte ageing.

In a mouse model, telomere shortening has also been observed in maternally aged oocytes but not in post-ovulatory-aged oocytes (Yamada-Fukunaga *et al.* 2013). The authors concluded that the long-term adverse effects of low telomerase activity and increased ROS exposure during reproductive ageing are likely associated with telomere shortening in oocytes from reproductive-aged females.

Oocyte ageing has also been reported to affect the DNA of the oocyte and induce epigenetic changes. Tarin *et al.* (2000) and Yamada-Fukunaga *et al.* (2013) proposed that the accumulation of oxidative damage of mitochondrial DNA, proteins and lipids in the aged oocytes might be responsible for the decreased viability rates and reduced potential for embryo development. Lipid oxidation products, such as lipid peroxyl and alkoxyl radicals, cause mitochondrial DNA damage via oxidation of bases and strand breaks (Combelles *et al.* 2009). The age-related decline in oocyte quality and chromosomal aneuploidy might result from mitochondrial damage by ROS produced over long periods (Tarin 1996; Combelles *et al.* 2009). Whitaker and Knight (2008) indicated that, whenever SOD was inhibited, DNA fragmentation increased, and that elevated glutathione peroxidase (GPx) and catalase (CAT) activities were accompanied by reduced DNA fragmentation. Liang *et al.* (2008) investigated the DNA methylation of differentially methylated regions of two maternal genes, *Snrpn* and *Peg1/Mest*, during the ageing of mouse oocytes. The authors reported the demethylation of *Snrpn* following ovulation in mouse oocytes in both *in vivo* and in culture medium *in vitro*. Following fertilization, demethylation events occur in parental genomes (Mayer *et al.* 2000), and ageing of the oocyte can alter the methylation pattern of genes in the oocyte (Liang *et al.* 2008). Therefore, an altered epigenetic profile, which would be able to change gene expression in embryos arising from *in vivo* and *in vitro* aged oocytes, might be responsible for some of the abnormalities observed in the later life of the offspring (Lord & Aitken 2013).

Molecular changes during the progress of oocyte ageing

Most findings regarding molecular changes during oocyte ageing have been obtained in studies of higher vertebrates, in which ageing has been associated with accumulation of molecular damage (Cefalu 2011). Comparatively few studies have analysed gene expression and egg quality associated with oocyte ageing in fish (Aegerter *et al.* 2005; Bonnet *et al.* 2007b; Ma *et al.* 2015). Any change in oxygen concentrations can stimulate expression of redox-sensitive transcription factors, such as HIF, that may up- or down-regulate target genes (Harvey *et al.* 2004). Additionally, the levels and processing of maternally provided mRNAs and proteins can be affected by non-genetic effects of environmental variables, including the oocyte ageing (Bonnet *et al.* 2007b). Information on gene expression changes during oocyte ageing in fish, as well as in higher vertebrates, is summarized below.

Genes involved in oxidative damage, stress responses and mitochondrial function

Very often, genes involved in oxidative stress and mitochondrial function show an upward trend in expression during oocyte ageing. Esponda and Diaz (2006) analysed the presence of *hsp70* mRNA and protein in samples of 20-h aged mice oocytes and found that Hsp70 protein is not detectable in freshly ovulated oocytes; however, this protein appears in the cytoplasm of the aged oocytes. They also reported increased *hsp70* mRNA levels in the aged oocytes. Similarly, upregulation of *cox1* in mouse oocytes undergoing maternal ageing has been reported (Hamatani *et al.* 2004). Increased expression of *cox1* in aged eggs may affect an abnormal mitochondrial respiratory chain and, therefore, impact egg quality. In our recent study, we observed the upregulation of *hsp70* and *cox1* mRNAs during post-ovulatory and post-stripping oocyte ageing in common carp *Cyprinus carpio* (Azin Mohagheghi Samarin, Azadeh Mohagheghi Samarin, Tone-Kari Knutsdatter Østbye, Bente Ruyter, Sabine Sampels, Viktoriia Burkina, Miroslav Blecha, David Gela & Tomas Policar, unpubl. data). The protein encoded by *cox1*, located in the mitochondrial membrane, is the last enzyme in the respiratory electron transport chain. Therefore, any changes in the mRNA expression pattern of *cox1* might affect ATP synthesis and induce mitochondrial dysfunction. ROS can directly affect the Ca^{2+} handling system. Additionally, oxidation affects calcium binding to calmodulin protein and leads to perturbed calcium homeostasis in human epidermal cells (Schallreuter *et al.* 2007). An impairment of Ca^{2+} reuptake by Ca^{2+} ATPases in the endoplasmic reticulum (ER) of aged mouse oocytes has been identified (Igarashi *et al.* 1997). Ca^{2+} release from the 1,4,5-triphosphate (InsP3)-sensitive Ca^{2+} stores decreases in aged mouse oocytes due to depletion of the ER Ca^{2+} store (Takahashi *et al.* 2000). In mouse oocytes aged *in vitro*, calcium oscillation changes during fertilization are characterized by a higher frequency but lower amplitude in aged oocytes (Takahashi *et al.* 2003, 2009). The molecular mechanism of this phenomenon is likely associated with impaired Ca^{2+} regulation (Takahashi *et al.* 2003). A higher amount of calcium has been observed in aged oocytes in rainbow trout *Oncorhynchus mykiss* and

Caspian brown trout *Salmo trutta caspius* (Craik & Harvey 1984; Bahrekazemi *et al.* 2010). Therefore, the increased frequency of calcium oscillations during fertilization in aged oocytes may be related to the elevated calcium levels. Following mitochondrial dysfunction and consequent ATP depletion, a decrease in the transcription levels of ATP-related genes is observed and the function of proteins that require ATP also decreases (Hamatani *et al.* 2004). Other oxidative stress-related genes, *sod* and *gpx*, are known to provide protection against oxidative damage. Alteration of antioxidant enzymes may decrease the capacity to cope with oxidative damage in oocytes over time following ovulation. Evaluation of the activity of antioxidant enzymes during oocyte ageing and correlating the results with the expression of related genes during this time seems to be a promising area of research in future studies.

In rainbow trout eggs, ova ageing also results in the downregulated expression of specific microRNAs and their target genes. These genes are mainly involved in cell death and signal transduction, stress response and DNA damage, RNA degradation, and energy and transcription regulation (Ma *et al.* 2015).

Genes involved in the cell cycle, DNA and chromosome stability

Regulation of genes involved in cell cycling has also been investigated with regard to post-ovulatory and post-stripping oocyte ageing. An increasing trend in the expression of *cyclin A1*, *cyclin A2* and *JNK1* has been reported in rainbow trout oocytes aged *in vivo* (Aegerter *et al.* 2005). Similarly, *Cyclin A*, *cyclin B*, *jnkA* and *jnkB* displayed higher expression in more aged oocytes *in vivo* in common carp, but lower expression in more aged oocytes *in vitro* (Azin Mohagheghi Samarin, Azadeh Mohagheghi Samarin, Tone-Kari Knutsdatter Østbye, Bente Ruyter, Sabine Sampels, Viktoriia Burkina, Miroslav Blecha, David Gela & Tomas Policar, unpubl. data). Interestingly, the peak relative abundances of *cyclin A1* and *A2* mRNAs in rainbow trout eggs are observed 5 days following ovulation, and not immediately at ovulation, synchronous with egg viability, which also peaks a few days after ovulation (Aegerter *et al.* 2005). Decreased mRNA levels of two critical cell cycle-related genes, maturation-promoting factor (MPF) and mitogen-activated protein kinases (*MAPKs*), have been reported in porcine oocytes aged *in vitro* (Kikuchi *et al.* 2002; Ma *et al.* 2005) and in mouse oocytes (Xu *et al.* 1997). The latter study indicated a role for critical cell cycle factors and cytoplasmic changes in spontaneous activation of oocyte ageing. The reasons for the observed opposite trend towards the up- or downregulation of the above-mentioned genes during ova ageing *in vivo* and *in vitro,* respectively, are a subject for future studies. However, it should be taken into the consideration that the oocyte ageing process occurs more quickly *in vitro* than it does *in vivo* (e.g. Azuma *et al.* 2003; Rizzo *et al.* 2003; Samarin *et al.* 2016). Therefore, the same trend in gene expression might be observed earlier *in vitro* compared to the *in vivo* storage condition.

Hamatani *et al.* (2004) suggested that decreased mitochondrial expression of ATP-related genes, and resulting decreased functioning of proteins dependent on ATP, might be critical factors affecting microtubule and cytoskeleton proteins leading to chromosome segregation defects and accompanying increase in aneuploidy in maternally aged oocytes. Microtubule and cytoskeleton proteins are likely important because malfunctions in these proteins cause failed chromosome segregation and an accompanying increase in aneuploidy, a major pathological phenotype of post-ovulatory-aged oocytes in several fish species (Hamatani *et al.* 2004). Ova ageing in rainbow trout (Aegerter & Jalabert 2004), tench *Tinca tinca* (Flajshans *et al.* 2007), pikeperch *Sander lucioperca* (Samarin *et al.* 2015a) and Northern pike *Esox lucius* (Samarin *et al.* 2016) has been associated with the increased occurrence of triploidization. During the ageing of pig and mouse oocytes, the expression of mitotic arrest deficient protein (*MAD2*) exhibits a downward trend (Ma *et al.* 2005; Steuerwald *et al.* 2005). The authors concluded that decreased levels of *MAD2* transcripts in aged mouse oocytes coincide with higher frequencies of premature centromere separation–complete (*PCS-C*), indicating that *MAD2* decline with oocyte ageing is responsible for chromosome missegregation and aneuploidy. Additionally, a compromised spindle assembly checkpoint (SAC) might be responsible for the reproductive failure observed as a consequence of oocyte ageing (Steuerwald *et al.* 2005). The decreased expression of *MAD2* mRNA has also been reported with maternal ageing in human oocytes (Steuerwald *et al.* 2001).

Furthermore, *in vitro* aged human oocytes show the aberrant expression of *g-tubulin*, indicating the disruption of the centrosome structure at the meiotic poles (Sun & Schatten 2007). Yamada-Fukunaga *et al.* (2013) observed that the transcript levels of *Tert*, a catalytic subunit of the enzyme telomerase, in post-ovulatory-aged mouse oocytes are significantly reduced compared with those in fresh oocytes. However, the authors reported that post-ovulatory ageing had no significant effect on the relative telomerase activity and relative telomere length of oocytes. It is noteworthy that the level of telomerase reverse transcriptase (*Tert*), whose expression decreases during general ageing, also decreases in maternally aged oocytes (Hamatani *et al.* 2004) as well as post-ovulatory-aged oocytes. This is consistent with the notion that short telomeres in the chromosomes of human eggs indicate a poor prognosis following *in vitro* fertilization embryo transfer (Keefe *et al.* 2003).

Genes involved in apoptosis

Apoptotic cell death is the end point of the oocyte ageing process that occurs through caspase activation (Takai *et al.* 2007), increased levels of apoptotic signalling protein *Bax*, decreased levels of anti-apoptotic protein *Bcl-xL* (Perez *et al.* 2005) and DNA damage (Fujino *et al.* 1996). We recently found that the genes involved in apoptosis, such as *caspase3A*, *caspase9* and *bax,* exhibited lower transcript levels at the time of ovulation than in over-ripened eggs in common carp (Azin Mohagheghi Samarin, Azadeh Mohagheghi Samarin, Tone-Kari Knutsdatter Østbye, Bente Ruyter, Sabine Sampels, Viktoriia Burkina, Miroslav Blecha, David Gela & Tomas Policar, unpubl. data). By contrast, it has been shown that the expression of *bax* remains unchanged in the mouse oocytes aged *in vitro* (Gordo *et al.* 2002; Takahashi *et al.* 2013). At the end of the oocyte ageing time, pro-apoptotic molecules, such as *bax*, induce the release of *cytochrome c*, which activates caspases, while anti-apoptotic molecules, such as *bcl2*, prevent this release (Li *et al.* 2000). Expression of the anti-apoptotic protein Bcl2 is decreased during oocyte ageing in mice (Gordo *et al.* 2002) and pigs (Ma *et al.* 2005), making the oocytes and developing embryos more prone to undergo apoptosis (Lord *et al.* 2013; Takahashi *et al.* 2013). As Bcl2 belongs to a family of proteins regulating cell death, some of which (e.g. Bax) are pro-apoptotic (Tsujimoto *et al.* 1984; Cleary *et al.* 1986), expression of *bcl2* is not as the only parameter controlling Bcl2 action and the consequent apoptosis.

In African catfish, *Clarias gariepinus,* the *in vitro* oocyte ageing induced by delayed fertilization resulted in an upward trend in the expression of *cathepsinD*, an apoptosis-related gene (Samarin *et al.* 2018). Lord *et al.* (2013) suggested that oxidative stress in aged oocytes can be considered as an early marker of oocyte ageing before the activation of caspase-3 and also before the appearance of the morphological features of oocyte ageing and apoptosis. Upregulation of *cathepsinB* is associated with cell death (Houseweart *et al.* 2003). Aegerter *et al.* (2005) also found that *in vivo* oocyte ageing in rainbow trout is associated with the upregulation of *cathepsinZ*. The lysosomal proteases, cathepsin D and cathepsin B, act as pro-apoptotic mediators of apoptosis (Kågedal *et al.* 2001). Therefore, the increased expression of the *cathepsinB* and *cathepsinZ* genes during the oocyte ageing progress might lead to the upregulation of the apoptotic genes. Moreover, an increase in cathepsin D activity can be considered as an early apoptosis marker before any other biochemical or morphological signs of apoptosis are evident. Cathepsins may also function at this time in ways unrelated to apoptosis, such as in digestion of yolk proteins for recycling.

Genes involved in fertilization, embryo development and transcriptional regulation

The expression of *vasa* has been shown to be downregulated with maternal ageing in mouse oocytes (Hamatani *et al.* 2004). *Vasa* is a gene involved in the development of primordial germ cells (PGCs), and its activity is required for differentiation of the germ cells into gametes (Raz 2000) as well as the functionality of germ cells (Nikolic *et al.* 2016). Loss of *vasa* function in the mouse affects differentiation of the male germ cells resulting in male sterility and lack of any male phenotypic sex characteristic in the knockout mice (Raz 2000). Vasa protein is an essential component of germplasm and represents a poorly understood complex of RNA and proteins that is required for germ cell determination. Null mutation leads to sterility in female mice resulting from severe defects in oogenesis (Saffman & Lasko 1999). Tarin *et al.* (2000) concluded that oocyte ageing is associated with distorted secondary sex ration in favour of males. The *vasa* expression and the relation to the number of PGCs in fish as well as the sex of the offspring can be addressed in the future studies.

Aegerter *et al.* (2005) found higher quantities of *igf2* mRNAs in more aged rainbow trout oocytes at 14 h post-ovulation compared to the freshly ovulated ones. The IGF axis plays roles in the inhibition of apoptotic cell death (Butt *et al.* 1999). The increased tendency towards the mRNA levels of the *igf2* therefore might be considered as a possible defence mechanism against the occurrence of over-ripening of the eggs and ensuing apoptosis. The mRNA expression of *nucleoplasmin* (*npm2*), which is considered to play an important role in the early stages of embryo development, was shown to decrease over time following ovulation in rainbow trout eggs (Aegerter *et al.* 2005).

The mRNA levels of *PGC-1a*, *NRF-1* and mitotic spindle checkpoint protein *SMAD2* (mothers against decapentaplegic homolog 2) were significantly lower in the aged than in non-aged ova, whereas the relative gene expression of the epigenetic modification gene *HAT1* (histone acetylation 1) and hyaluronan synthase gene *HAS3* (hyaluronan synthase 3) exhibited an increase as the ova culture was prolonged (Zhang *et al.* 2013). The spindle checkpoint protein, *SMAD2,* regulates viability and developmental ability in mammalian oocytes (Wang *et al.* 2007). Therefore, the age-dependent loss in the function of the spindle checkpoint system may result in defects in the developmental competence of the oocytes (Zhang *et al.* 2013). In addition, the authors concluded that changes in the mRNA expression levels of genes related to ageing in cumulus cells could be an effective alternative for the detection of oocyte ageing. In fish, the larval malformation rates increase significantly with ova ageing (e.g. Aegerter & Jalabert 2004;

Bonnet *et al.* 2007a; Samarin *et al.* 2017). The involvement of *sox9* in human skeletal malformation syndrome has been confirmed (Foster *et al.* 1994; Wagner *et al.* 1994) and, therefore, dysregulation of *sox9a* may represent at least one of the causes of such malformations in fish larvae.

It is believed that control of development by maternal genes switches to control by zygotic genes during the mid-blastula transition in fish embryos (Tadros & Lipshitz 2009). As transcription of the maternal genome is silent from prophase I of the first meiotic cell division up to zygotic genome activation, cytoplasmic regulation of mRNA adenylation is an important mechanism of post-transcriptional control in oocytes (Paynton *et al.* 1988). Until zygotic genome activation, protein levels can be regulated by the poly-A tail length of maternal gene transcripts (Clarke 2012; Weill *et al.* 2012). Delayed fertilization in aged oocytes may affect this post-transcriptional regulation, followed by developmental defects. Oocyte ageing in murine metaphase II oocytes was recently shown to be associated with shortening of the poly-A tails of maternal effect genes both *in vivo* and *in vitro* (Dankert *et al.* 2014). The authors concluded that poly-A tail shortening can, in turn, affect the time of protein translation of maternal gene transcripts, followed by disturbed fertilization and developmental defects. Moreover, a study in frog *Xenopus tropicalis* indicated that the 3-h post-ovulatory ageing of oocytes leads to deadenylation of transcripts involved in translation and energy metabolism, a consequent decrease in fertilization rates and an increase in mortality and malformations (Kosubek *et al.* 2010).

Oocyte ageing significantly alters the methylation pattern of imprinted genes in both mouse oocytes and the developing placenta (Liang *et al.* 2008). This, in turn, alters the demethylation events after fertilization (Mayer *et al.* 2000). The altered epigenetic profile can change gene expression in embryos obtained from aged oocytes. As oocyte manipulation can effect on the risk of aberrant methylation (Anifandis *et al.* 2015), DNA methylation status of the embryos originating from the aged oocytes deserves to be examined in the future studies. Histone acetylation and DNA methylation may lead to gene expression changes without modifying the genome. Hamatani *et al.* (2004) reported that genes encoded in the mitochondrial genome were more highly expressed in old oocytes. By contrast, genes encoded in the nuclear genome were more highly expressed in young oocytes. Therefore, the origin of the selected genes may also affect their expression.

Delaying or preventing oocyte ageing

The hypothesis suggesting the involvement of oxidative stress as the initiator for ova ageing is growing in acceptance (e.g. Tarin *et al.* 2000; Takahashi *et al.* 2003; Lord *et al.* 2013). According to this hypothesis, antioxidant treatment of oocytes might prevent or delay the ageing process. To prevent or delay oocyte ageing, treating oocyte culture media with antioxidant and non-antioxidant compounds, such as caffeine in pigs (Kikuchi *et al.* 2002), nitric oxide (Goud *et al.* 2005) and DL-dithiothreitol in mice (Rausell *et al.* 2007) and humans (Tarin *et al.* 1998) and trichostatin A in pigs (Jeseta *et al.* 2008) and mice (Huang *et al.* 2007), has been applied. Antioxidants can reduce the incidence of aneuploidy in aged oocytes (Eichenlaub-Ritter 2012). The supplementation of aged mouse oocyte culture media with 1 mM of the potent antioxidant hormone melatonin results in a 41% decrease in ROS levels, delayed apoptosis and the prevention of DNA fragmentation (Lord *et al.* 2013). More importantly, this supplementation with melatonin could broaden the time window for successful fertilization and even improve resulting embryo quality (Lord *et al.* 2013). In addition, the supplementation of the oocyte culture media with caffeine, a phosphodiesterase inhibitor, leads to increased fertilization rate (Ono *et al.* 2011); however, no relief from oxidation and apoptosis was achieved. Furthermore, oocyte treatment with caffeine did not improve embryo quality (Lord *et al.* 2013). Caffeine can prevent the inactivation of critical cell cycle factors, particularly MPF (Kikuchi *et al.* 2002), and reduce calcium release defects (Zhang *et al.* 2011). In addition, caspase activation was significantly reduced only in the presence of melatonin in the oocyte culture media, or when caffeine was used together with melatonin, suggesting that melatonin is a more effective and safer compound than caffeine to delay the oocyte ageing process (Lord *et al.* 2013).

If oxidative stress is the initiator for the undesirable consequences of oocyte ageing, increased expression of antioxidant enzymes and of enzymes involved in the repair of oxidative damage may be expected in freshly ovulated oocytes following fertilization. A recent study suggested an increased capacity to cope with oxidative insults in mouse oocytes post-fertilization (Lord & Aitken 2013). Additionally, the expression of genes involved in the protection against oxidative damage and stress responses was decreased in oocytes during reproductive ageing (Hamatani *et al.* 2004). The effect of antioxidants and oxidative damage on oocyte ageing and over-ripening in fish has not yet been examined (Samarin *et al.* 2015b) but appears to be a potentially valuable topic for future research.

Conclusions

Post-ovulatory oocyte ageing has been identified as the most important factor affecting egg quality in several fish species and is associated with deleterious effects. Oocyte ageing is associated with reduced fertilization success,

degraded embryo quality and increased abnormalities observed in the later life of the offspring, which may involve epigenetic changes. The exact mechanisms underlying the oocyte ageing process are still unclear. Most authors have suggested that increased ROS and oxidative stress are initiators of oocyte ageing. The major consequences are ROS-induced mitochondrial dysfunction, lipid alterations, DNA fragmentation and chromosomal abnormalities. Mitochondrial dysfunction leads to decreased ATP production and consequently impaired Ca^{2+} oscillation during fertilization. A reduction in membrane fluidity is observed following the occurrence of lipid peroxidation in the oocyte membrane that might cause decreased fusion of sperm and oolemma, resulting in a decreased fertilization rate in aged oocytes. Oocyte ageing has also been reported to affect the DNA of the oocyte and induce epigenetic changes. Apoptotic cell death is the endpoint of these deleterious effects. In higher vertebrates, treatment of unfertilized eggs with antioxidants has been shown to delay, prevent or even reverse the ageing process during *in vitro* storage.

Unfertilized ovulated oocytes contain maternal mRNAs that direct the early stages of embryogenesis until the zygotic transcription is activated. Several studies have demonstrated that oocyte ageing is associated with the accumulation of molecular damage including altered mRNA expression profiles in eggs of fish and other model animals. Identifying mRNAs and proteins that are differentially expressed during fish oocyte ageing and discovering the underlying mechanisms of gene and protein regulation would contribute information valuable to basic research and for practical applications in aquaculture to delay or even prevent post-ovulatory oocyte ageing. Evaluation of the activity of antioxidant enzymes during oocyte ageing and correlating the results to the expression of related genes during this time also seems to be a promising path for future studies. No study has yet been performed on the effect of fish oocyte ageing on the subsequent growth and genetic structure of progeny. This topic also should be addressed in future studies. Additionally, as the demand for assisted reproduction technology is increasing and very few studies in this field are available, a comparative study on the process of oocyte ageing in fish might be beneficial to research on higher vertebrates. In addition, almost nothing is known of 'maternal oocyte ageing' in fish, which might be an excellent model for studying the phenomenon.

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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**: 59–71.

Aegerter S, Jalabert B, Bobe J (2005) Large scale real-time PCR analysis of mRNA abundance in rainbow trout eggs in relationship with egg quality and post-ovulatory ageing. *Molecular Reproduction and Development* **72**: 377–385.

Anifandis G, Messini CI, Dafopoulos K, Messinis IE (2015) Genes and conditions controlling mammalian pre- and post-implantation embryo development. *Current Genomics* **16**: 32–46.

Azuma T, Ohta H, Oda S, Muto K, Yada T, Unuma T (2003) Changes in fertility of rainbow trout eggs retained in coelom. *Fisheries Science (Tokyo)* **69**: 131–136.

Bahrekazemi M, Soltani M, Matinfar A, Abtahi B, Pusti I, Samarin AM *et al.* (2010) Biochemical and histological studies of over-ripened oocyte in the Caspian brown trout (*Salmo trutta caspius*) to determine biomarkers for egg quality. *Iranian Journal of Fisheries Sciences* **9**: 33–48.

Bobe J, Labbe C (2010) Egg and sperm quality in fish. *General and Comparative Endocrinology* **165**: 535–548.

Boerjan ML, 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**: 155–163.

Bonnet E, Fostier A, Bobe J (2007a) Characterization of rainbow trout egg quality: a case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations. *Theriogenology* **67**: 786–794.

Bonnet E, Fostier A, Bobe J (2007b) Microarray-based analysis of fish egg quality after natural or controlled ovulation. *BMC Genomics* **8**: 55.

Butt AJ, Firth SM, Baxter RC (1999) The IGF axis and programmed cell death. *Immunology and Cell Biology* **77**: 256–262.

Cefalu CA (2011) Theories and mechanisms of aging. *Clinics in Geriatric Medicine* **27**: 491–506.

Chian RC, Nakahara H, Niwa K, Funahashi H (1992) Fertilization and early cleavage in vitro of ageing bovine oocytes after maturation in culture. *Theriogenology* **37**: 665–672.

Clarke HJ (2012) Post-transcriptional control of gene expression during mouse oogenesis. *Results and Problems in Cell Differentiation* **55**: 1–21.

Cleary ML, Smith SD, Sklar J (1986) Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* **47**: 19–28.

Combelles CMH, Gupta S, Agarwal A (2009) Could oxidative stress influence the in-vitro maturation of oocytes? *Reproductive Biomedicine Online* **18**: 864–880.

Craik JCA, Harvey SM (1984) Egg quality in rainbow trout: the relation between egg viability, selected aspects of egg composition, and time of stripping. *Aquaculture* **40**: 115–134.

Dankert D, Demond H, Trapphoff T, Heiligentag M, Rademacher K, Eichenlaub-Ritter U *et al.* (2014) Pre- and postovulatory aging of murine oocytes affect the transcript level and poly(A) tail length of maternal effect genes. *PLoS One* **9**: e108907.

Eichenlaub-Ritter U (2012) Oocyte ageing and its cellular basis. *The International Journal of Developmental Biology* **56**: 841–852.

Esponda P, Diaz H (2006) Age-induced apoptosis and activation of heat shock protein HSP70 in mammalian oocytes. *FEBS Journal* **273**: 368–371.

Flajshans M, Kohlmann K, Rab P (2007) Autotriploid tench *Tinca tinca* (L.) larvae obtained by fertilization of eggs previously subjected to postovulatory ageing *in vitro* and *in vivo*. *Journal of Fish Biology* **71**: 868–876.

Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M *et al.* (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* **372**: 525–530.

Fujino Y, Ozaki K, Yamamasu S, Ito F, Matsuoka I, Hayashi E *et al.* (1996) DNA fragmentation of oocytes in aged mice. *Human Reproduction* **11**: 1480–1483.

Gordo AC, Rodrigues P, Kurokawa M (2002) Intracellular calcium oscillations signal apoptosis rather than activation in *in vitro* aged mouse eggs. *Biology of Reproduction* **66**: 1828–1837.

Goud AP, Goud PT, Diamond MP, Abu-Soud HM (2005) Nitric oxide delays oocyte aging. *Biochemistry* **44**: 11361–11368.

Hall VJ, Compton D, Stojkovic P, Nesbitt M, Herbert M, Murdoch A *et al.* (2007) Developmental competence of human *in vitro* aged oocytes as host cells for nuclear transfer. *Human Reproduction* **22**: 52–62.

Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA *et al.* (2004) Age-associated alteration of gene expression patterns in mouse oocytes. *Human Molecular Genetics* **13**: 2263–2278.

Harvey AJ, Kind KL, Pantaleon M, Armstrong DT, Thompson JG (2004) Oxygen-regulated gene expression in bovine blastocysts. *Biology of Reproduction* **71**: 1108–1119.

Houseweart MK, Pennacchio LA, Vilaythong A, Peters C, Noebels JL, Myers RM (2003) Cathepsin B but not cathepsins L or S contributes to the pathogenesis of Unverricht-Lundborg progressive myoclonus epilepsy (EPM1). *Journal of Neurobiology* **56**: 315–327.

Huang JC, Yan LY, Lei ZL, Miao YL, Shi LH, Yang JW *et al.* (2007) Changes in histone acetylation during postovulatory aging of mouse oocyte. *Biology of Reproduction* **77**: 666–670.

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

Jeseta M, Petr J, Krejcova T, Chmelikova E, Jilek F (2008) *In vitro* ageing of pig oocytes: effects of the histone deacetylase inhibitor trichostatin A. *Zygote* **16**: 145–152.

Jing L, Li Q, He L, Sun W, Jia Z, Ma H (2017) Protective effect of tempol against hypoxia-induced oxidative stress and apoptosis in H9c2 cells. *Medical Science Monitor Basic Research* **23**: 159–165.

Kågedal K, Johansson U, Öllinger K (2001) The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. *Federation of American Societies for Experimental Biology* **15**: 1592–1594.

Keefe D, Franco S, Liu L, Trimarchi J, Blasco M, Weitzen S (2003) Short telomeres in the chromosomes of spare eggs predict poor prognosis following *in vitro* fertilization/embryo transfer—toward a telomere theory of reproductive aging in women. *Fertility and Sterility* **80** (Suppl 3): S1.

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 and Stem Cells* **4**: 211–222.

Kosubek A, Klein-Hitpass L, Rademacher K, Horsthemke B, Ryffel GU (2010) Aging of *Xenopus tropicalis* eggs leads to deadenylation of a specific set of maternal mRNAs and loss of developmental potential. *PLoS One* **5**: e13532.

Lahnsteiner F (2000) Morphological, physiological and biochemical parameters characterizing the over-ripening of rainbow trout eggs. *Fish Physiology and Biochemistry* **23**: 107–118.

Lahnsteiner F, Urbanyi B, Horvath A, Weismann T (2001) Biomarkers for egg quality determination in cyprinid fish. *Aquaculture* **195**: 331–352.

Lam TJ, Nagahama Y, Chan K, Hoar WS (1978) Overripe eggs and post-ovulatory corpora lutea in the three spined stickleback. *Canadian Journal of Zoology* **56**: 2029–2036.

Li M, Baumeister P, Roy B, Phan T, Foti D, Luo S *et al.* (2000) ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. *Molecular and Cellular Biology* **20**: 5096–5106.

Liang XW, Zhu JQ, Miao YL, Liu JH, Wei L, Lu SS *et al.* (2008) Loss of methylation imprint of Snrpn in postovulatory aging mouse oocyte. *Biochemical and Biophysical Research Communications* **371**: 16–21.

Liang XW, Ge ZI, Wei L, Guo L, Han ZM, Schatten H *et al.* (2011) The effects of postovulatory aging of mouse oocytes on methylation and expression of imprinted genes at midterm gestation. *Molecular Human Reproduction* **17**: 562–567.

Liu L, Keefe DL (2000) Cytoplasm mediates both development and oxidation-induced apoptotic cell death in mouse zygotes. *Biology of Reproduction* **62**: 1828–1834.

Lord T, Aitken RJ (2013) Oxidative stress and ageing of the post-ovulatory oocyte. *Reproduction* **146**: 217–227.

Lord T, Nixon B, Jones KT, Aitken RJ (2013) Melatonin prevents postovulatory oocyte aging in the mouse and extends the window for optimal fertilization *in vitro*. *Biology of Reproduction* **88**: 1–9.

Ma W, Zhang D, Hou Y, Li YH, Sun QY, Sun XF *et al.* (2005) Reduced expression of MAD2, BCL2, and MAP kinase activity in pig oocytes after *in vitro* aging are associated with defects in sister chromatid segregation during meiosis II and embryo fragmentation after activation. *Biology of Reproduction* **72**: 373–383.

Ma H, Weber GM, Hostuttler MA, Wei H, Wang L, Yao J (2015) MicroRNA expression profiles from eggs of different qualities associated with post-ovulatory ageing in rainbow trout (*Oncorhynchus mykiss*). *BMC Genomics* **16**: 201.

Mayer W, Nivelelai A, Walter J, Fundele R, Haaf T (2000) Demethylation of the zygotic paternal genome. *Nature* **403**: 501–502.

Minami N, Suzuki T, Tsukamoto S (2007) Zygotic gene activation and maternal factors in mammals. *The Journal of Reproduction and Development* **53**: 707–715.

Mok HJ, Shin H, Lee JW, Lee GK, Suh CS, Kim KP *et al.* (2016) Age-associated lipidome changes in metaphase II mouse oocytes. *PLoS One* **11**: e0148577.

Nikolic A, Volarevic V, Armstrong L, Lako M, Stojkovic M (2016) Primordial germ cells: current knowledge and perspectives. *Stem Cells International* **2016**: 1741072.

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**: 460–471.

Ott M, Gogvadze V, Orrenius S, Zhivotovsky B (2007) Mitochondria, oxidative stress and cell death. *Apoptosis* **12**: 913–922.

Paynton BV, Rempel R, Bachvarova R (1988) Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. *Developmental Biology* **129**: 304–314.

Perez GI, Juriscova A, Matikainen T, Morityama T, Kim MR, Takai Y *et al.* (2005) A central role for ceramide in the age-related acceleration of apoptosis in the female germline. *Federation of American Societies for Experimental Biology* **19**: 860–862.

Rausell F, Pertusa JF, Gomez-Piquer V, Hermenegildo C, Garcia-Perez MA, Cano A *et al.* (2007) Beneficial effects of dithiothreitol on relative levels of glutathione S-transferase activity and thiols in oocytes, and cell number, DNA fragmentation and allocation at the blastocyst stage in the mouse. *Molecular Reproduction and Development* **74**: 860–869.

Raz E (2000) The function and regulation of vasa-like genes in germ-cell development. *Genome Biology* **1** (3): reviews 1017.1–1017.6.

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**: 26–36.

Rizzo E, Godinho HP, Sato Y (2003) Short-term storage of oocytes from the neotropical teleost fish *Prochilodus marggravii*. *Theriogenology* **60**: 1059–1070.

Saffman E, Lasko P (1999) Germline development in vertebrates and invertebrates. *Cellular and Molecular Life Sciences* **55**: 1141–1163.

Samarin AM, Mojazi Amiri B, Soltani M, Nazari RM, Kamali A, Naghavi MR (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**: 12309–12314.

Samarin AM, Gela D, Bytyutskyy D, Policar T (2015a) Determination of the best post-ovulatory stripping time for the common carp, *Cyprinus carpio*. *Journal of Applied Ichthyology* **31**: 51–55.

Samarin AM, Policar T, Lahnsteiner F (2015b) Fish oocyte ageing and its effect on egg quality. *Reviews in Fisheries Science & Aquaculture* **23**: 302–314.

Samarin AM, Blecha M, Uzhytchak M, Bytyutskyy D, Żarski D, Flajshans M *et al.* (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 AM, Żarski D, Palińska-Żarska K, Krejszeff S, Blecha M, Kucharczyk D *et al.* (2017) In vitro storage of unfertilized eggs of the Eurasian perch and its effect on egg viability rates and the occurrence of larval malformations. *Animal* **11**: 78–83.

Samarin AM, Sampels S, Policar T, Rodina M, Hematyar N, Samarin AM (2018) mRNA abundance changes during *in vitro* oocyte ageing in African catfish *Clarias gariepinus* (Burchell, 1822). *Aquaculture Research* **49**: 1037–1045.

Sanchez AM, Valeria Stella Vanni VS, Bartiromo L, Papaleo E, Zilberberg E, Candiani M *et al.* (2017) Is the oocyte quality affected by endometriosis? A review of the literature. *Journal of Ovarian Research* **10**: 43.

Schallreuter KU, Gibbons NC, Zothner C, Abou Elloof MM, Wood JM (2007) Hydrogen peroxide-mediated oxidative stress disrupts calcium binding on calmodulin: more evidence for oxidative stress in vitiligo. *Biochemical and Biophysical Research Communications* **360**: 70–75.

Simsek-Duran F, Li F, Ford W, Swanson RJ, Jones HW, Castora FJ (2013) Age-associated metabolic and morphologic changes in mitochondria of individual mouse and hamster oocytes. *PLoS One* **8**: e64955.

Steuerwald N, Cohen J, Herrera RJ, Sandalinas M, Brenner CA (2001) Association between spindle assembly checkpoint expression and maternal age in human oocytes. *Molecular Human Reproduction* **7**: 49–55.

Steuerwald NM, Steuerwald MD, Mailhes JB (2005) Post-ovulatory aging of mouse oocytes leads to decreased MAD2 transcripts and increased frequencies of premature centromere separation and anaphase. *Molecular Human Reproduction* **11**: 623–630.

Sun QY, Schatten H (2007) Centrosome inheritance after fertilization and nuclear transfer in mammals. *Advances in Experimental Medicine and Biology* **591**: 58–71.

Tadros W, Lipshitz HD (2009) The maternal-to-zygotic transition: a play in two acts. *Development* **136**: 3033–3042.

Tafani M, Sansone L, Limana F, Arcangeli T, De Santis E, Polese M *et al.* (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, Saito H, Hiroi M, Doi K, Takahashi E (2000) Effects of aging on inositol 1,4,5-triphosphate-induced Ca (2+) release in unfertilized mouse oocytes. *Molecular Reproduction and Development* **55**: 299–306.

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* **66**: 143–152.

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**: 493–502.

Takahashi T, Igarashi H, Amita M, Hara S, Kurachi H (2011) Cellular and molecular mechanisms of various types of oocyte aging. *Reproductive Medicine and Biology* **10**: 239–249.

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. *The Journal of Obstetrics and Gynaecology Research* **39**: 1431–1439.

Takai Y, Matikainen T, Juriscova A, Kim MR, Trbovich AM, Fujita E *et al.* (2007) Caspase-12 compensates for lack of caspase-2 and caspase-3 in female germ cells. *Apoptosis* **12**: 791–800.

Tarin JJ (1996) Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Molecular Human Reproduction* **2**: 717–724.

Tarin JJ, Vendrell FJ, Ten J, Cano A (1998) Antioxidant therapy counteracts the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes. *Molecular Human Reproduction* **4**: 281–288.

Tarin JJ, Perez-Albala S, Cano A (2000) Consequences on offspring of abnormal function in ageing gametes. *Human Reproduction Update* **6**: 532–549.

Tarin JJ, Perez-Albala S, Perez-Hoyos P, Cano A (2002) Postovulatory aging of oocytes decreases reproductive fitness and longevity of offspring. *Biology of Reproduction* **66**: 495–499.

Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* **226**: 1097–1099.

Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J *et al.* (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* **79**: 1111–1120.

Wakayama S, Thuan NV, Kishigami S, Ohta H, Mizutani E, Hikichi T *et al.* (2004) Production of offspring from one-day-old oocytes stored at room temperature. *Journal of Reproduction and Development* **50**: 627–637.

Wang JY, Lei ZL, Nan CL, Yin S, Liu J, Hou Y *et al.* (2007) RNA interference as a tool to study the function of MAD2 in mouse oocyte meiotic maturation. *Molecular Reproduction and Development* **74**: 116–124.

Weill L, Belloc E, Bava FA, Mendez R (2012) Translational control by changes in poly(A) tail length: recycling mRNAs. *Nature Structural & Molecular Biology* **19**: 577–585.

Wesson DE, Elliott SJ (1995) The H2O2-generating enzyme, xanthine oxidase, decreases luminal Ca2+ content of the IP3-sensitive Ca2+ store in vascular endothelial cells. *Microcirculation* **2**: 195–203.

Whitaker BD, Knight JW (2008) Mechanisms of oxidative stress in porcine oocytes and the role of antioxidants. *Reproduction Fertility and Development* **20**: 694–702.

Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML *et al.* (2001) Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Human Reproduction* **16**: 909–917.

Xu Z, Abbott A, Kopf GS, Schultz RM, Ducibella T (1997) Spontaneous activation of ovulated mouse eggs: time-dependent effects on M-phase exit, cortical granule exocytosis, maternal messenger ribonucleic acid recruitment, and inositol 1,4,5-triphosphate sensitivity. *Biology of Reproduction* **57**: 743–750.

Yamada-Fukunaga T, Yamada M, Hamatani T, Chikazawa N, Ogawa S, Akutsu H *et al.* (2013) Age-associated telomere shortening in mouse oocytes. *Reproductive Biology and Endocrinology* **11**: 108.

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS (1998) Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Human Reproduction* **13**: 998–1002.

Yoshida M, Ishigati K, Nagai T, Chikyu M, Pursel VG (1993) Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. *Biology of Reproduction* **49**: 89–94.

Yoshida N, Brahmajisyula M, Shoji S, Amanai M, Perry AC (2007) Epigenetic discrimination by mouse metaphase II oocytes mediated asymmetric chromatin remodelling independently of meiotic exit. *Developmental Biology* **301**: 464–477.

Zhang N, Wakai T, Fissore RA (2011) Caffeine alleviates the deterioration of Ca (2C) release mechanisms and fragmentation of *in vitro*-aged mouse eggs. *Molecular Reproduction and Development* **78**: 684–701.

Zhang GM, Gu CH, Zhang YL, Sun HY, Qian WP, Zhou ZR *et al.* (2013) Age-associated changes in gene expression of goat oocytes. *Theriogenology* **80**: 328–336.

CHAPTER 2

mRNA ABUNDANCE CHANGES DURING *IN VITRO* OOCYTE AGEING IN AFRICAN CATFISH *CLARIAS GARIEPINUS* (BURCHELL, 1822)

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mRNA abundance changes during in vitro oocyte ageing in African catfish *Clarias gariepinus* (Burchell, 1822)

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Abstract

Delayed fertilization following ovulation leads to the oocyte ageing which has been identified as the most important factor affecting egg quality after ovulation. Very little is known about the molecular changes associated with the progress of oocyte ageing. The present study monitored the egg viability rates during post-stripping oocyte ageing in African catfish *Clarias gariepinus*. In addition the mRNA abundance of selected genes were studied during the progress of oocyte ageing by real time quantitative PCR. To study how maternal transcripts influence egg quality, expression levels were correlated with egg hatching rates. The highest embryo survival and hatching rates (88% and 81%, respectively) were obtained from eggs that were fertilized immediately after stripping. Complete loss of egg viability occurred at 16 and 24 hr Post Stripping (HPS) when eggs were stored at 25°C and 4°C respectively. Under both storage temperatures, the embryo mortality and larval malformation rates increased significantly over time and were the highest in the most aged oocytes. Genes indicating an upward trend in expression during ova ageing were determined to be related to oxidative injury and the stress response (*hsp70*), mitochondrial function (*calmodulin*), apoptosis (*cathepsin D*) and germ line speciation (*vasa* and *sox9a*). The results suggest that oxidative stress and mitochondrial dysfunction might be associated with post-stripping ova ageing and the consequent induction of egg quality defects. The examined genes may be considered as candidate markers of egg quality associated with oocyte ageing in African catfish.

KEYWORDS
Clarias gariepinus, egg quality, gene expression, oocyte ageing

1 | INTRODUCTION

Ovulated oocytes are arrested at the metaphase II stage of meiotic division and await fertilization. Any delay in egg spawning and/or stripping leads to excessive oocyte ageing and ultimately egg overripening. Decreased egg quality has been documented in several fish species following prolonged ova ageing (Rizzo, Godinho & Sato, 2003). Ova ageing in fish is associated with a limited fertilization rate (e.g., Lahnsteiner, Urbanyi, Horvath & Weismann, 2001; Samarin, Gela, Bytyutskyy & Policar, 2015), increased larval malformations (e.g., Aegerter & Jalabert, 2004; Bonnet, Fostier & Bobe, 2007a; Samarin et al., 2017) increased ploidy anomalies (e.g., Flajshans, Kohlmann & Rab, 2007; Samarin et al., 2016) and, in higher vertebrates, poor embryo development (Chian, Nakahara, Niwa & Funahashi, 1992) and offspring abnormalities through epigenetic changes (e.g., Liang, Zhu & Miao, 2008; Tarin, Perez-Albala, Perez-Hoyos &

Cano, 2002). The optimal time period for oocyte fertilization differs among fish species and the egg storage temperature, and varies from a few minutes to a few weeks (reviewed by Samarin, Policar & Lahnsteiner, 2015). In most fish species, egg storage at higher temperatures results in a much faster and stronger decrease in egg quality compared to lower temperatures (e.g., Aegerter & Jalabert, 2004; Samarin et al., 2008, 2017).

Until now, many studies have focused on the morphological, physiological, biochemical and histological changes that occur inside eggs during oocyte ageing (for details see Samarin, Policar et al., 2015). However, little is known regarding the molecular changes associated with this process in fish. Few studies have analysed the gene expression and egg quality associated with oocyte ageing in fish (Aegerter, Jalabert & Bobe, 2005; Bonnet, Fostier & Bobe, 2007b; Ma et al., 2015; Mommens et al., 2010). Early stages of embryonic development are highly dependent on maternally provided mRNAs and proteins (Bobe & Labbe, 2010). Previous studies on higher vertebrates have suggested a role for oxidative stress in the unfavourable reproductive outcomes associated with oocyte ageing (e.g., Liang et al., 2008; Miao, Kikuchi, Sun & Schatten, 2009). These studies report that oxidative stress can, in turn, trigger many cascades affecting oocyte quality, such as mitochondrial dysfunction, DNA damage, perturbed Ca^{2+} homeostasis and lipid peroxidation. Age-associated alterations in gene expression during mice and human oocyte ageing have also identified candidate mRNAs and proteins involved in the spindle assembly checkpoint and regulation and control of the cell cycle, particularly those related to mitochondrial function and energy pathways (Hamatani et al., 2004; Steuerwald, Bermudez, Wells, Munne & Cohen, 2007). However, the specific molecular functions that determine egg quality associated with oocyte ageing in fish and in higher vertebrates remain to be elucidated.

Fish are good model animals to evaluate oocyte ageing, as they display a vast diversity of reproductive modes and most produce a high number of oocytes compared to other animals. Therefore, influences by other factors affecting oocyte quality, including female age, feeding, water temperature and water composition, are excluded. The aim of the present study was to examine the transcriptional trend of African catfish *Clarias gariepinus* ova at varying ageing times in vitro up to the egg over-ripening stage and complete loss of fertilizing ability. The artificial reproduction of African catfish, a commercially important species, is easy and can be induced several times per year. The mRNA levels of genes involved in oxidative damage, mitochondrial function and the stress response (*hsp70*, *cox1* and *calmodulin*), fertilization and germ line (*vasa* and *sox9a*) and apoptosis (*cathepsin D*) was examined. The relationships between mRNA levels and success of hatching were also examined.

2 | MATERIALS AND METHODS

2.1 | Ethics

All experimental trials were conducted in accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals of the Czech Republic. The protocol was approved by the Committee on the Ethics of Animals at the University of South Bohemia in Ceske Budejovice.

2.2 | Fish

Brood African catfish (body weight 3 – 4 kg) were cultured under controlled conditions of a recirculating aquaculture system at experimental facilities of the South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic and used for the following experiment. Five days before hormonal treatment, five females and five males were moved to experimental cylindrical-shaped tanks (each 450 L capacity) under the following conditions: water temperature 23 ± 0.5°C, pH 7.0 ± 0.2, oxygen saturation 65 ± 10%, and light regime 12 L:12 D with the intensity of 35 lx. Each female was stocked separately to one tank.

Before hormonal treatment, females were anaesthetized with a clove oil bath (30 mg/L) (Kristan et al., 2014) and ovulation was induced with Ovopel preparation (Interfish Ltd., Hungary) (dosage 20 μg 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). Male brood fish were not treated hormonally. After hormonal treatment, females were examined for ovulation every hour beginning 12 hr after injection. The occurrence of ovulation was judged according to the method described by Samarin et al. (2017). All five females, which ovulated within 1 hr, were used for the experiment. To examine ovulation and for egg collection, females were anaesthetized with the abovementioned treatment. To obtain testicular sperm required for artificial fertilization, males were manually sacrificed and the dissected testes stored at 10°C.

2.3 | In vitro egg storage in ovarian fluid

Stripped ova of five females were collected separately and stored in 6-well sterile cell culture plates (each well diameter: 3.5 cm). No solution, artificial media or extender was used during egg storage and therefore the eggs were maintained in ovarian fluid. All plates were individually covered with lids and stored in the dark at two different temperatures (4 and 25°C) for 24 hr. For storage at 4°C, the plates were transferred to a domestic refrigerator with an adjusted constant temperature. A laboratory incubator was used for storing eggs at 25°C. Stored ova were fertilized at 0 (immediately after stripping), 2, 4, 8, 16 and 24 HPS.

2.4 | Artificial fertilization

Sperm motility was assessed empirically for each of the five males separately before egg insemination by observing sperm under a microscope at a magnification of ×40 using a glass slide without a coverslip. To provide a uniform fertilizing ability for all egg batches, 0.5 ml of milt was collected separately from each of the five sacrificed males and pooled, and a total volume of 2.5 ml was used at each fertilization step. Each batch of 2-g eggs was separately

fertilized using 0.2 ml of mixed milt followed by the addition of 2 ml of hatchery water. In the preliminary tests, this ratio was confirmed to be sufficient to fertilize all eggs. Egg stickiness was removed by stirring the eggs for 45 min in a 1:6 solution of milk (3.5% fat):hatchery water.

2.5 | Examining egg developmental success and larval quality

Each batch of 2-g eggs (equivalent to ca. 1560 eggs) was placed into a separate small rectangular-shaped incubator (4.5-L capacity) with recirculating water at 23 ± 0.5°C and a flow rate of 1 L/min through each incubator. At each sampling time, egg quality was evaluated by measuring embryo survival, hatching, embryo mortality and larval malformation rates. Embryo survival rates were measured 24 hr after fertilization using a stereomicroscope (Nikon SMZ745T, Japan). Hatching rates were calculated 48 hr after fertilization by counting the number of embryos that reached the hatched-larvae-stage to the number of initially fertilized eggs. The embryo mortality percentages were defined as the number of dead eggs among the total number of eggs possessing embryos inside. Malformed larvae (e.g., spinal cord torsion, yolk sac, eye deformations, etc.) were counted 48 hr after fertilization using a stereomicroscope to determine the incidence of malformation rates.

2.6 | Sample collection for real-time qPCR analysis

At each HPS, 1 g of eggs was collected from each female (in three replicates) and placed into cryotubes (Thermo Fisher Scientific, USA). Thereafter, the samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA isolation.

2.7 | RNA isolation and reverse transcription

To prepare total RNA, an equal weight of 20 eggs were taken from each cryotube, treated with TRI reagent (Sigma-Aldrich, USA) and homogenized for 2 × 2 miFn with a 1-min interval using an Ultra Turrax homogenizer. RNA was then isolated using a spin column kit (RNeasy, QIAGEN). During RNA isolation, samples were depleted of genomic DNA by treatment with PureLink DNase (Invitrogen). RNA integrity was visualized by gel electrophoresis, and the concentration and purity were assessed using a NanoDrop Spectrophotometer (ND-1000, Thermo Scientific). Total RNA (1 μg) was used to generate cDNA with M-MLV Reverse Transcriptase (Promega). The reaction was performed at 70°C for 5 min, 4°C for 15 min and 37°C for 60 min. Control reactions were run in the absence of M-MLV Reverse Transcriptase and used as negative controls.

2.8 | Primer design and real-time qPCR analysis

Nucleotide sequences corresponding to African catfish mRNA (NCBI) were used for primer design (by Primer3) (Koressaar & Remm, 2007; Untergasser et al., 2012). The transcript levels of 6 genes (*hsp70*, *cox 1*, *calmodulin*, *vasa*, *cathepsin D* and *sox9*) were determined by real-time qPCR (BIO-RAD, CFX, USA). The reaction mixture for qPCR consisted of 5 μl of diluted (1:10) cDNA, 0.6 μl of forward and reverse primers (final concentration of 200 nM, Table 1), and 7.5 μl of SYBR Green PCR Master Mix (Roche) and was evaluated according to the standard curve method. All primers were provided by Sigma-Aldrich. All samples were analysed in parallel, and a non-template control with water substituted for cDNA was run for each primer pair. qPCR was performed under the following conditions: 95°C for 10 min, amplification with 40 cycles at 95°C for 15 s and 60°C for 1 min, melting curve at 95°C for 30 s and 70°C for 5 s, and cooling at 40°C for 10 s. The comparative CT method was used for relative quantification of target gene expression levels. mRNA expression data were normalized to that of *18S*. To normalize relative gene expression, two reference genes, *18S* ribosomal RNA (*18S*) and beta-actin (b-actin), were tested using RefFinder. The mRNA abundance of *18S* was highly stable in African catfish eggs collected at different time points after ovulation and the Ct values of the eggs at different HPSs did not change significantly. Therefore, *18S* was proven as the most stable gene and used as the reference. The relative expression was calculated according to the equation $2^{-\Delta Ct}$. Sequences of primers used are listed in Table 1.

2.9 | Statistical analysis

The normality of the data was assessed using SPSS software version 18. Differences between the means among groups for each measured egg quality parameter (*i.e.*, embryo survival, hatching, embryo

TABLE 1 GenBank accession numbers and primer sequences of genes studied for qRT-PCR

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Genbank accession no.
hsp 70	ATGAACCCCACCAACACAAT	ATGACCTTGAAAGGCCAATG	DQ885945
cox I	TTGTTACTGCACACGCCTTC	TCAGGAGCCCCTAGCATTAG	KF742418
vasa	AGGTGCAGGAGCCTGAAGTA	ACGCACACAAGTGCCATAAG	GU562470
sox9a	ACCCAGAGGCCCATGTACAC	CTGGTGGAGGGAGTGGAC	HM149258
b-actin	AGGATCTGTACGCCAACACC	GGGATGTGATCTCCTTCTGC	KJ722167
calmodulin	AGAGTTGGGGACTGTCATGC	TCTATCGTCCCATTCCCATC	KF892532
18s	GGAGTCTGGATGGATGGTGT	TGAGAACGGAGAGAGGGAGA	GQ465237
cathepsin D	TCTGGAAGTCTCTCCGGCTA	GTCATTCCAGGCTGCTTGAT	KF014001

mortality and larval malformation rates), as well as each individual gene, were evaluated using ANOVA followed by Duncan's multiple range test. $p < .05$ was considered to indicate significance. The success of hatching was correlated with the mRNA expression profiles during oocyte ageing for each HPS.

3 | RESULTS

3.1 | Egg viability and larval malformation rates during in vitro ova ageing

The embryo survival and hatching rates were 88% and 81%, respectively, for eggs fertilized immediately after stripping. Thereafter, the values decreased significantly over time and reached 0% at 16 HPS after storage at 25°C (Figure 1). After 24 hr of storage at 4°C, the embryo survival rates measured 3% (Figure 2).

At both storage temperatures, the embryo mortality and larval malformation rates increased significantly in aged eggs. At the storage temperature of 4°C, the values measured 63% and 21%, respectively, at 8 HPS (Figure 2). 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 (Figure 1). Spinal cord torsion was found to be the most common type of the hatched larvae malformation.

3.2 | mRNA abundance trends during ova ageing in vitro

The mRNA levels of 6 selected transcripts were quantified and compared in freshly ovulated and post-stripping aged oocytes using *18S* as the reference gene. When eggs were stored at 25°C, the relative abundance of *hsp70*, *calmodulin*, *cathepsin D*, *vasa*, *sox9a* and *cox1* to *18S* exhibited an upward trend up to 8 hr of egg storage and a downward trend up to 12 HPS, except for *cox1*, which stayed almost constant after 8 HPS (Figure 3). At the storage temperature of 4°C, the mRNA abundance of *hsp70*, *cathepsin D* and *vasa* exhibited an upward trend up to 12 HPS and a downward trend thereafter. The levels of *calmodulin* and *sox9a*, however, were slightly upregulated and *cox1* was slightly downregulated during ova ageing. None of the

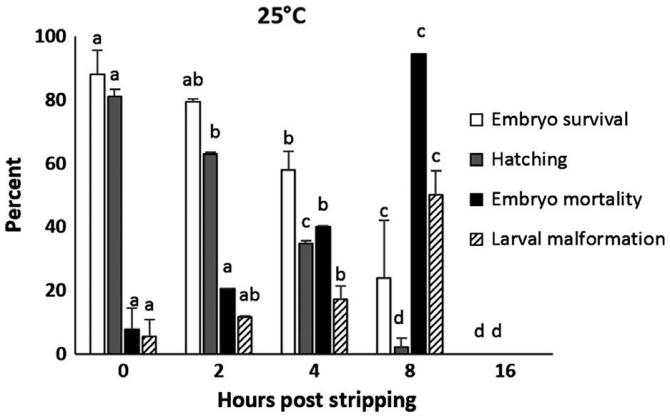

FIGURE 1 Effects of post-stripping ova ageing on embryo survival, hatching, embryo mortality and larval malformation rates of African catfish eggs stored at 4°C (mean ± *SD*) (n = 5 females). Means sharing a common alphabetical symbol do not differ significantly among the different HPS under the investigated parameters

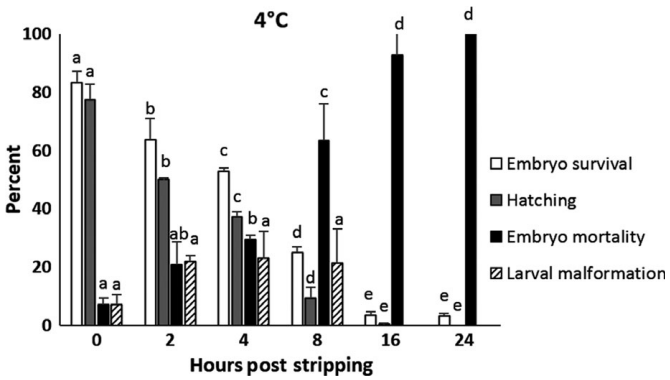

FIGURE 2 Effects of post-stripping ova ageing on embryo survival, hatching, embryo mortality and larval malformation rates of African catfish eggs stored at 25°C (mean ± *SD*) (n = 5 females). Means sharing a common alphabetical symbol do not differ significantly among the different HPS under the investigated parameters

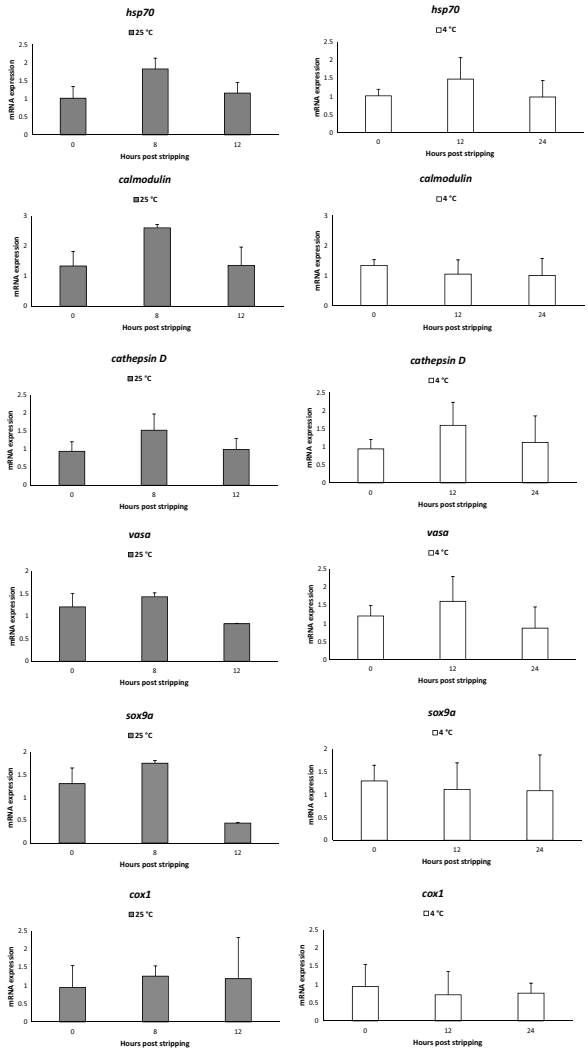

FIGURE 3 Effects of *in vitro* oocyte ageing at 25°C and 4°C on the mRNA expression levels of selected genes in African catfish (mean ± *SD*) (n = 5 females)

abovementioned genes exhibited statistically significant difference in their relative abundance during the ova ageing

3.3 | Correlations between egg hatching rate and mRNA levels of selected genes during ova ageing in vitro

The strongest negative correlations ($R^2 > 0.9$) were observed between egg hatching rate and the mRNA expression profile of *cox1* when eggs were stored at 25°C (Table 2), and *cox1*, *calmodulin* and *sox9a* when eggs were stored at 4°C (Table 3).

4 | DISCUSSION

Under both storage temperatures, the embryo survival and hatching rates decreased, and embryo mortality and larval malformation percentages increased with time after stripping. This is in accordance

with the results obtained by Baidya and Senoo (2004), who found that the hatching rates of African catfish eggs were completely lost when eggs were stored for 6 hr at 27-28°C and all larvae from eggs at 6–8 HPS were malformed. In another strain of African catfish, *Heterobranchus longifilis*, no hatching of normal larvae took place after 8 HPS (Nguenga, Teugels, Legendre & Ollevier, 2004). The decreasing egg quality throughout oocyte ageing observed in this study is also in accordance with results obtained from previous experiments performed on other fish species (for details see Samarin, Policar et al., 2015). However, the time period after ovulation during which eggs retain their fertilizing ability significantly depends on the fish species and egg storage temperature as well as the temperature preference of the fish species.

Lower egg storage temperatures usually guarantee longer successful storage time compared to the higher temperatures (e.g., Azuma et al., 2003; Samarin et al., 2008, 2017). However, in 2 fish species, turbot (*Psetta maxima*) (Suquet et al., 1999) and curimata (*Prochilodus marggravii*) (Rizzo et al., 2003), it has been shown that the storage of their eggs at temperatures that the fish do not experience during their normal range of life results in a decrease in the egg viability.

The molecular mechanisms involved in the process of ova ageing have been poorly studied in fish. In higher vertebrates, however, increasing reactive oxygen species (ROS) and consequent oxidative stress has been proposed to be the initiating factor for the progress of oocyte ageing (Lord & Aitken, 2013; Takahashi, Takahashi, Igarashi, Tezuka & Kurachi, 2003; Tarin, Perez-Albala & Cano, 2000). As a result of increasing ROS, mitochondrial oxidative stress increases which, in turn, leads to mitochondrial dysfunction (Takahashi et al., 2003). In the current study, genes involved in "oxidative stress and mitochondrial function", namely, *hsp70*, *cox1* and *calmodulin*, showed an upward trend in mRNA levels during oocyte ageing. A previous study performed by Esponda and Diaz (2006) also indicated increased expression of *hsp70* in post-ovulatory aged mouse oocytes compared to freshly ovulated ones. These authors analysed the presence of *hsp70* protein and mRNA in oocyte samples collected from 20 HPO and reported that *hsp70* is not present in fresh oocytes; however, this protein appears in the cytoplasm of post-ovulatory aged oocytes. In aged human oocytes, increased ROS and oxidative stress contribute to mitochondrial dysfunction (Steuerwald et al., 2007). Therefore, it is likely that mitochondria play an important role in the oocyte ageing process. *cox*, a mitochondrial gene, is involved in stress responses and catalyses electron transfer from reduced cytochrome c to oxygen in the respiratory chain. In the present study, the mitochondria-related gene expression levels of *cox1* increased in oocytes with prolonged storage. Increased expression of *cox1* in aged eggs may cause an abnormal mitochondrial respiratory chain and therefore affect egg quality. Similarly, upregulation of *cox1* in mouse oocytes with maternal ageing has been reported (Hamatani et al., 2004). In another study, we observed the upregulation of *hsp70* and *cox1* mRNAs during post-ovulatory and post-stripping oocyte ageing in common carp *Cyprinus carpio* (unpublished data). These preliminary results indicate the possible involvement of the oxidative stress on the progress of oocyte ageing in African catfish. Evaluation of the activity of antioxidant enzymes during oocyte ageing seems to be a promising area of research in future studies.

In the present study, *calmodulin* mRNA level exhibited an upward trend during oocyte ageing. Reactive oxygen species can directly affect the Ca^{2+} handling system. Additionally, oxidation affects calcium binding to the calmodulin protein and leads to perturbed calcium homeostasis in human epidermal cells (Schallreuter, Gibbons, Zothner, Abou Elloof & Wood, 2007). An impairment of Ca^{2+} reuptake by Ca^{2+} ATPases in the endoplasmic reticulum (ER) of aged mouse oocytes has been identified (Igarashi, Takahashi, Hiroi & Doi, 1997). Ca^{2+} release from the 1,4,5- triphosphate (InsP3)-sensitive Ca^{2+} stores decreases in aged mouse oocytes due to depletion of the ER Ca^{2+} store (Takahashi, Saito, Hiroi, Doi & Takahashi, 2000). In mouse oocytes aged in vitro, calcium oscillation changes during fertilization are characterized by a higher frequency but lower amplitude in aged oocytes (Takahashi et al., 2003, 2009). The molecular mechanism of this phenomenon is likely associated with impaired Ca^{2+} regulation (Takahashi et al., 2003). These authors indicated that altered Ca^{2+} oscillation leads to poor embryo development by affecting the formation of oocyte development. On the other hand, a higher amount of calcium has been observed in aged oocytes in rainbow trout *Oncorhynchus mykiss* and Caspian brown trout *Salmo trutta caspius* (Bahrekazemi et al., 2010; Craik & Harvey, 1984). Therefore, the increased frequency of calcium oscillations during fertilization in aged oocytes may be related to the higher amount of calcium that has been reported in aged oocytes. Following mitochondrial dysfunction and consequent ATP depletion, the function of proteins that require ATP also decreases (Hamatani et al., 2004). As such, the microtubule and cytoskeleton proteins are likely important because malfunctions in these proteins cause failed chromosome segregation and an accompanying increase in aneuploidy—a major pathological phenotype of post-ovulatory aged oocytes in several fish species (Hamatani et al., 2004). Ova ageing in rainbow trout (Aegerter & Jalabert, 2004), tench (Flajshans et al., 2007), pikeperch (Samarin, Blecha, Bytyutskyy & Policar, 2015) and Northern pike (Samarin et al., 2016) has been associated with the increased occurrence of triploidization.

The increased tendency of *vasa* mRNA level observed in this study is consistent with the results obtained in another recent experiment by our research group, indicating that the expression of *vasa* mRNA increases during oocyte ageing in common carp (unpublished data), although its expression is downregulated with maternal ageing in mouse oocytes (Hamatani et al., 2004). *Vasa* is involved in reproduction (Hamatani et al., 2004) and is expressed in germ cells, specifically the germline stem cells (GSCs) of female ovaries. In zebrafish, *vasa* RNA is important for early determination of the germ plasm, and its role in the development of germ cells in several other vertebrates has been evidenced (Raz, 2000). Conversely, Tarin et al. (2000) indicated that oocyte ageing is associated with distorted secondary sex ration in favour of males. Therefore, it can be postulated that *vasa* is involved in sex determination of the offspring, although this hypothesis should be addressed in future studies. Another gene, *sox9*, exhibited a tendency of upregulation during ova ageing in

TABLE 2 Regression model for the gene expression associated with hatching rates at different hours post-stripping as the variable parameter in African catfish eggs stored at 25°C

Target gene	Regression model	R^2
hsp 70	y = −0.0059x + 1.5002	0.394
cox1	y = −0.0035x + 1.2269	0.948
calmodulin	y = −0.008x + 1.9909	0.253
vasa	y = 0.001x + 1.1274	0.023
Sox9a	y = 0.0028x + 1.0874	0.036
cathepsin D	y = −0.0039x + 1.2629	0.310

TABLE 3 Regression model for the gene expression associated with hatching rates at different hours post-stripping as the variable parameter in African catfish eggs stored at 4°C

Target gene	Regression model	R^2
hsp 70	y = −0.0025x + 1.2288	0.157
cox1	y = 0.0027x + 0.7341	0.943
calmodulin	y = 0.0042x + 1.0149	0.995
vasa	y = 2E-05x + 1.2282	4E-06
Sox9a	y = 0.0027x + 1.0962	0.996
cathepsin D	y = −0.0052x + 1.3631	0.454

African catfish. The involvement of *sox9* in human skeletal malformation syndrome has been confirmed (Foster et al., 1994; Wagner et al., 1994). In the current study, the larval malformation rates increased significantly with ova ageing, so that under the egg storage temperature of 25°C, approximately 50% of the hatched larvae were malformed. Therefore, it can be proposed that upregulation of *sox9a* may represent at least one of the stimulators in the occurrence of malformed larvae.

In this study, the in vitro oocyte ageing induced by delayed fertilization resulted in an upward trend in the mRNA level of *cathepsin D*, an apoptosis-related gene. Apoptotic cell death is the end point of the oocyte ageing process. Aegerter et al. (2005) also found that in vivo oocyte ageing in rainbow trout is associated with the upregulation of *cathepsin Z*. Lysosomal proteases, such as *cathepsin D* and *cathepsin B*, act as pro-apoptotic mediators (Kågedal, Johansson & Öllinger, 2001). Moreover, an increase in *cathepsin D* activity can be considered an early apoptosis marker before any biochemical or morphological signs of apoptosis.

It is believed that the transition from maternal genes to the zygotic genome occurs during the mid-blastula in fish embryo development (Tadros & Lipshitz, 2009). Since transcription of the maternal genome is silent from prophase I of the first meiotic cell division up to zygotic genome activation, cytoplasmic regulation of mRNA adenylation is an important mechanism of post-transcriptional control in oocytes (Paynton, Rempel & Bachvarova, 1988). Until zygotic genome activation, protein levels can be regulated by the poly-A tail length of maternal genes (Clarke, 2012; Weill, Belloc, Bava & Mendez, 2012). Delayed fertilization in aged oocytes may affect this post-transcriptional regulation, followed by developmental defects. Oocyte ageing in murine metaphase II oocytes was recently shown to be associated with shortening of the poly-A tails of maternal effect genes both in vivo and in vitro (Dankert et al., 2014). The authors concluded that poly-A tail shortening can, in turn, affect the time of protein translation of maternal gene transcripts, followed by disturbed fertilization and developmental defects. Moreover, a study in frog *Xenopus tropicalis* indicated that the 3- hr post-ovulatory ageing of oocytes leads to deadenylation of transcripts involved in translation and energy metabolism, a consequent decrease in fertilization rates and an increase in mortality and malformations (Kosubek, Klein-Hitpass, Rademacher, Horsthemke & Ryffel, 2010). In the current study, expression of the selected genes, and not the related proteins, changed during oocyte ageing. Upregulation of several transcripts during oocyte ageing has also been indicated in several previous studies (Aegerter et al., 2005; Dankert et al., 2014; Zhang et al., 2013). Aegerter et al. (2005) reported that since real-time PCR provides information only on the relative amount of gene expression standardized to the abundance of the reference gene, it is possible that the time-dependent increase in transcripts could be due to higher degradation of the reference gene. However, even in such cases, a lower upregulation rate of the selected genes with a concurrently higher downregulation rate of the reference gene remains a possibility. In contrast, choosing the most stable reference gene would not be convincing. In the current experiment, highly stable expression of *18S* was observed during oocyte ageing. Hence, the possibility of the results being affected by the higher degradation rate of the reference gene is omitted. Dankert et al. (2014) also reported that the *Brg1* and *Oct4* genes showed an increase in total transcript levels after aging in comparison to controls and mentioned that they do not have an explanation for it yet as oocytes should be transcriptionally inactive. Oocyte ageing significantly alters the methylation pattern of imprinted genes in both mouse oocytes and the developing placenta (Liang et al., 2008). This, in turn, alters the demethylation events after fertilization (Mayer, Nivelelai, Walter, Fundele & Haaf, 2000). The altered epigenetic profile can change gene expression in embryos obtained from aged oocytes. Histone acetylation and DNA methylation may lead to gene expression changes without modifying the genome. Therefore, the increasing trend of the expression of several genes observed in our study may be attributed to the effect of oocyte ageing as an epigenetic factor through its impact on DNA methylation or histone acetylation. Hamatani et al. (2004) reported that genes encoded in the mitochondrial genome were more highly expressed in old oocytes. By contrast, genes encoded in the nuclear genome were more highly expressed in young oocytes. Therefore, the origin of the selected genes may also affect their expression before fertilization so that mitochondrial-related genes are expressed during oocyte ageing.

5 | CONCLUSION

The results obtained in this study demonstrate that maternal mRNA abundance may be affected by the ova ageing time and altered

following a prolonged time interval between ovulation and fertilization. Oxidative stress and mitochondrial dysfunction seems to be associated with post-stripping ova ageing in African catfish and the consequent induction of egg quality defects.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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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*, 59–71. https://doi.org/10.1016/j.aquaculture.2003.08.019

Aegerter, S., Jalabert, B., & Bobe, J. (2005). Large scale real-time PCR analysis of mRNA abundance in rainbow trout eggs in relationship with egg quality and postovulatory ageing. *Molecular Reproduction and Development*, *72*, 377–385. https://doi.org/10.1002/(ISSN)1098-2795

Azuma, T., Ohta, H., Oda, S., Muto, K., Yada, T., & Unuma, T. (2003). Changes in fertility of rainbow trout eggs retained in coelom. *Fisheries Science*, *69*, 131–136. https://doi.org/10.1046/j.1444-2906.2003.00597.x

Bahrekazemi, M., Soltani, M., Matinfar, A., Abtahi, B., Pusti, I., Mohagheghi Samarin, A., & Mojazi Amiri, B. (2010). Biochemical and histological studies of over-ripened oocyte in the Caspian brown trout (Salmo trutta caspius) to determine biomarkers for egg quality. *Iranian Journal of Fisheries Sciences*, *9*(1), 33–48.

Baidya, A. P., & Senoo, S. (2004). Decline in fertilization and hatching rates of African catfish clarias gariepinus after ovulation. *Suisanzoshoku*, *52*(1), 37–44.

Bobe, J., & Labbe, C. (2010). Egg and sperm quality in fish. *General and Comparative Endocrinology*, *165*, 535–548. https://doi.org/10.1016/j.ygcen.2009.02.011

Bonnet, E., Fostier, A., & Bobe, J. (2007a). Characterization of rainbow trout egg quality: A case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations. *Theriogenology*, *67*, 786–794. https://doi.org/10.1016/j.theriogenology.2006.10.008

Bonnet, E., Fostier, A., & Bobe, J. (2007b). Microarray-based analysis of fish egg quality after natural or controlled ovulation. *BMC Genomics*, *8*, 55. https://doi.org/10.1186/1471-2164-8-55

Chian, R. C., Nakahara, H., Niwa, K., & Funahashi, H. (1992). Fertilization and early cleavage in vitro of ageing bovine oocytes after maturation in culture. *Theriogenology*, *37*, 665–672. https://doi.org/10.1016/0093-691X(92)90146-I

Clarke, H. J. (2012). Post-transcriptional control of gene expression during mouse oogenesis. *Results and Problems in Cell Differentiation*, *55*, 1–21.

Craik, J. C. A., & Harvey, S. M. (1984). Biochemical changes associated with overripening of the eggs of rainbow trout, Salmo gairdneri. *Aquaculture*, *37*, 347–357. https://doi.org/10.1016/0044-8486(84)90299-0

Dankert, D., Demond, H., Trapphoff, T., Heiligentag, M., Rademacher, K., Eichenlaub-Ritter, U., … Grummer, R. (2014). Pre- and postovulatory aging of murine oocytes affect the transcript level and poly(A) tail length of maternal effect genes. *PLoS ONE*, *9*(10), e108907. https://doi.org/10.1371/journal.pone.0108907

Esponda, P., & Diaz, H. (2006). Age-induced apoptosis and activation of heat shock protein HSP70 in mammalian oocytes. *FEBS Journal*, *273*, 368–371. https://doi:10.1111/j.1742-4658.2006.05313.x

Flajshans, M., Kohlmann, K., & Rab, P. (2007). Autotriploid tench *Tinca tinca* (L.) larvae obtained by fertilization of eggs previously subjected to postovulatory ageing *in vitro* and *in vivo*. *Journal of Fish Biology*, *71*, 868–876. https://doi.org/10.1111/jfb.2007.71.issue-3

Foster, J. W., Dominguez-Steglich, M. A., Guioli, S., Kwok, C., Weller, P. A., Stevanovic, M., … Schafer, A. J. (1994). Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature*, *372*, 525–530. https://doi.org/10.1038/372525a0

Hamatani, T., Falco, G., Carter, M. G., Akutsu, H., Stagg, C. A., Sharov, A. A., … Ko, M. S. H. (2004). Age-associated alteration of gene expression patterns in mouse oocytes. *Human Molecular Genetics*, *13*(19), 2263–2278. https://doi.org/10.1093/hmg/ddh241

Igarashi, H., Takahashi, E., Hiroi, M., & Doi, K. (1997). Aging-related changes in calcium oscillations in fertilized mouse oocytes. *Molecular Reproduction and Development*, *48*, 383–390. https://doi.org/10.1002/(ISSN)1098-2795

Kågedal, K., Johansson, U., & Öllinger, K. (2001). The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. *Faseb Journal*, *15*, 1592–1594.

Koressaar, T., & Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics*, *23*(10), 1289–1291. https://doi.org/10.1093/bioinformatics/btm091

Kosubek, A., Klein-Hitpass, L., Rademacher, K., Horsthemke, B., & Ryffel, G. U. (2010). Aging of Xenopus tropicalis eggs leads to deadenylation of a specific set of maternal mRNAs and loss of developmental potential. *PLoS ONE*, *5*(10), e13532. https://doi.org/10.1371/journal.pone.0013532

Kristan, J., Stara, A., Polgesek, M., Drasovean, A., Kolarova, J., Priborsky, J., … Velisek, J. (2014). Efficacy of different anaesthetics for pike-perch (Sander lucioperca L.) in relation to water temperature. *Neuro Endocrinology Letters*, *35*(Suppl 2), 81–85.

Lahnsteiner, F., Urbanyi, B., Horvath, A., & Weismann, T. (2001). Bio-markers for egg quality determination in cyprinid fish. *Aquaculture*, *195*, 331–352. https://doi.org/10.1016/S0044-8486(00)00550-0

Liang, X. W., Zhu, J. Q., & Miao, Y. L. (2008). Loss of methylation imprint of Snrpn in postovulatory aging mouse oocyte. *Biochemical and Biophysical Research Communications*, *371*, 16–21. https://doi.org/10.1016/j.bbrc.2008.03.105

Lord, T., & Aitken, R. J. (2013). Oxidative stress and ageing of the post-ovulatory oocyte. *Reproduction*, *146*, 217–227. https://doi.org/10.1530/REP-13-0111

Ma, H., Weber, G. M., Hostuttler, M. A., Wei, H., Wang, L., & Yao, J. (2015). MicroRNA expression profiles from eggs of different qualities associated with post-ovulatory ageing in rainbow trout

(Oncorhynchus mykiss). *BMC Genomics*, *16*, 201. https://doi.org/10.1186/s12864-015-1400-0

Mayer, W., Nivelelai, A., Walter, J., Fundele, R., & Haaf, T. (2000). Demethylation of the zygotic paternal genome. *Nature*, *403*, 501–502. https://doi.org/10.1038/35000656

Miao, Y. L., Kikuchi, K., Sun, Q. Y., & Schatten, H. (2009). Oocyte aging: Cellular and molecular changes, developmental potential and reversal possibility. *Human Reproduction Update*, *15*, 573–585. https://doi.org/10.1093/humupd/dmp014

Mommens, M., Fernandes, J. M., Bizuayehu, T. T., Bolla, S. L., Johnston, I. A., & Babiak, I. (2010). Maternal gene expression in Atlantic halibut (Hippoglossus hippoglossus L.) and its relation to egg quality. *BMC Research Notes*, *3*, 138. https://doi.org/10.1186/1756-0500-3-138

Nguenga, D., Teugels, G. G., Legendre, M., & Ollevier, F. (2004). Effects of storage and incubation temperature on the viability of eggs, embryos and larvae in two strains of an African catfish, *Heterobranchus longifilis* (Siluriformes, Clariidae). *Aquaculture Research*, *35*, 1358–1369. https://doi.org/10.1111/are.2004.35.issue-14

Paynton, B. V., Rempel, R., & Bachvarova, R. (1988). Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. *Developmental Biology*, *129*, 304–314. https://doi.org/10.1016/0012-1606(88)90377-6

Raz, E. (2000). The function and regulation of vasa-like genes in germ-cell development. *Genome Biology*, *1*(3). reviews1017.

Rizzo, E., Godinho, H. P., & Sato, Y. (2003). Short-term storage of oocytes from the neotropical teleost fish *Prochilodus marggravii*. *Theriogenology*, *60*, 1059–1070. https://doi.org/10.1016/S0093-691X(03)00108-0

Samarin, A. M., Ahmadi, M. R., Azuma, T., Rafiee, G. R., Mojazi Amiri, B., & Naghavi, M. R. (2008). Influence of the time to egg stripping on eyeing and hatching rates in rainbow trout *Oncorhynchus mykiss* under cold temperatures. *Aquaculture*, *278*, 195–198. https://doi.org/10.1016/j.aquaculture.2008.03.034

Samarin, A., Blecha, M., Bytyutskyy, D., & Policar, T. (2015). Post-ovulatory oocyte ageing in pikeperch (*Sander lucioperca* L.) and its effect on egg viability rates and the occurrence of larval malformations and ploidy anomalies. *Turkish Journal of Fisheries and Aquatic Sciences*, *15*, 435–441.

Samarin, A. M., Blecha, M., Uzhytchak, M., Bytyutskyy, D., Żarski, 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. https://doi.org/10.1016/j.aquaculture.2015.08.017

Samarin, A., Gela, D., Bytyutskyy, D., & Policar, T. (2015). Determination of the best post-ovulatory stripping time for the common carp (*Cyprinus carpio* Linnaeus, 1758). *Journal of Applied Ichthyology*, *31*(Suppl. 2), 51–55. https://doi.org/10.1111/jai.2015.31.issue-s2

Samarin, A. M., Policar, T., & Lahnsteiner, F. (2015). Fish oocyte ageing and its effect on egg quality. *Reviews in Fisheries Science & Aquaculture*, *23*, 302–314. https://doi.org/10.1080/23308249.2015.1053560

Samarin, A. M., Żarski, D., Palińska-Żarska, K., Krejszeff, S., Blecha, M., Kucharczyk, D., & Policar, T. (2017). In vitro storage of unfertilized eggs of the Eurasian perch and its effect on egg viability rates and the occurrence of larval malformations. *Animal*, *11*(1), 78–83. https://doi.org/10.1017/S1751731116001361

Schallreuter, K. U., Gibbons, N. C., Zothner, C., Abou Elloof, M. M., & Wood, J. M. (2007). Hydrogen peroxide-mediated oxidative stress disrupts calcium binding on calmodulin: More evidence for oxidative stress in vitiligo. *Biochemical and Biophysical Research Communications*, *360*(1), 70–75. https://doi.org/10.1016/j.bbrc.2007.05.218

Steuerwald, N. M., Bermudez, M. G., Wells, D., Munne, S., & Cohen, J. (2007). Maternal age-related differential global expression profiles observed in human oocytes. *Reproductive Biomedicine Online*, *14*, 700–708. https://doi.org/10.1016/S1472-6483(10)60671-2

Suquet, M., Chereguini, O., Omnes, M. H., Rasines, I., Normant, Y., Souto, I. P., & Quemener L. (1999). Effect of temperature, volume of ova batches, and addition of a diluent, an antibiotic, oxygen and a protein inhibitor on short-term storage capacities of turbot, Psetta maxima, ova. *Aquatic Living Resources*, *12*(4), 239–246.

Tadros, W., & Lipshitz, H. D. (2009). The maternal-to-zygotic transition: A play in two acts. *Development*, *136*, 3033–3042. https://doi.org/10.1242/dev.033183

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*, 493–502. https://doi.org/10.1095/biolreprod.108.072017

Takahashi, T., Saito, H., Hiroi, M., Doi, K., & Takahashi, E. (2000). Effects of aging on inositol 1,4,5-triphosphate-induced Ca(2 +) release in unfertilized mouse oocytes. *Molecular Reproduction and Development*, *55*, 299–306. https://doi.org/10.1002/(ISSN)1098-2795

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*, *66*, 143–152. https://doi.org/10.1002/(ISSN)1098-2795

Tarin, J. J., Perez-Albala, S., & Cano, A. (2000). Consequences on offspring of abnormal function in ageing gametes. *Human Reproduction Update*, *6*, 532–549. https://doi.org/10.1093/humupd/6.6.532

Tarin, J. J., Perez-Albala, S., Perez-Hoyos, P., & Cano, A. (2002). Postovulatory aging of oocytes decreases reproductive fitness and longevity of offspring. *Biology of Reproduction*, *66*, 495–499. https://doi.org/10.1095/biolreprod66.2.495

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3 - new capabilities and interfaces. *Nucleic Acids Research*, *40*(15), e115. https://doi.org/10.1093/nar/gks596

Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., . . . Schere, G. (1994). Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell*, *79*, 1111–1120. https://doi.org/10.1016/0092-8674(94)90041-8

Weill, L., Belloc, E., Bava, F. A., & Mendez, R. (2012). Translational control by changes in poly(A) tail length: Recycling mRNAs. *Nature Structural & Molecular Biology*, *19*, 577–585. https://doi.org/10.1038/nsmb.2311

Zhang, G. M., Gu, C. H., Zhang, Y. L., Sun, H. Y., Qian, W. P., Zhou, Z. R., . . . Wang, F. (2013). Age-associated changes in gene expression of goat oocytes. *Theriogenology*, *80*, 328–336. https://doi.org/10.1016/j.theriogenology.2013.04.019

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CHAPTER 3

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)

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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)

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Abstract

The effect of in vitro storage of oocytes on embryos survival rate, egg oxidation status, antioxidant enzyme activities, lipid classes and fatty acid composition profile was investigated in common tench *Tinca tinca*. In order to identify the role of oxidative stress in the progress of oocyte ageing the levels of thiobarbituric acid reactive substances (TBARS) and carbonyls as indicators of lipid and protein oxidation were measured and the activity of antioxidant enzymes were examined. Stripped ova from six females were stored in cell culture plates at 20°C for up to 10 hr post-stripping (HPS). The stored ova were fertilized and the embryo survival rates were assessed. The results indicated that tench eggs could be successfully stored in vitro for 4 hr after stripping at 20°C. Superoxide dismutase enzyme activity increased at 6 HPS, whereas glutathione peroxidase and glutathione reductase activities decreased in oocytes during in vitro storage ($p < .05$) at 4 and 8 HPSs, respectively. The level of malondialdehyde did not show any significant changes during the progress of oocyte ageing. Carbonyls increased up to 2 HPS and thereafter decreased significantly. However, ova ageing did not affect the main lipid class composition and the fatty acid composition of the eggs. Lower quality eggs exhibited lower levels of cholesterol but higher levels of triacylglycerol.

KEYWORDS
antioxidant enzymes, egg quality, oocyte ageing, oxidative stress, *Tinca tinca*

1 | INTRODUCTION

The quality of fish eggs is dependent on several intrinsic and environmental factors (Bobe & Labbe, 2010). Oocyte ageing, which refers to the time period between ovulation and fertilization, is identified as the most important factor affecting the egg quality of several fish species after ovulation (eg, McEvoy, 1984; Rime et al., 2004). During oocyte ageing, major morphological, physiological, biochemical, histological, cellular and molecular changes occur inside the eggs (for details see Samarin, Policar, & Lahnsteiner, 2015). These changes deteriorate the quality of the ovulated eggs and lead to a limited fertilization rate (eg, Lahnsteiner, Urbanyi, Horvath, & Weismann, 2001; Samarin et al., 2011), increased larval malformation (eg, Aegerter & Jalabert, 2004; Bonnet, Fostier, & Bobe, 2007; Rime et al., 2004) and increased ploidy anomalies (eg, Flajshans, Kohlmann, & Rab, 2007; Samarin et al., 2016). The time period

during which the oocytes retain their fertilizing ability varies from a few minutes to a few days and highly depends on the fish species and the storage temperature (Samarin et al., 2016).

Until now, there has been only a poor understanding about the processes and underlying mechanism of oocyte ageing in fish as well as in higher vertebrates. Since oxidative stress is a prominent mediator of ageing and disease in many cell and tissue types (Harman, 1988), most authors suggest that an increase in reactive oxygen species (ROS) might be an initiating factor for the progress of oocyte ageing in human (Lord & Aitken, 2013; Takahashi et al., 2013) and mouse oocytes (Lord, Nixon, Jones, & Aitken, 2013). Increasing ROS leads to increased oxidative stress and consequently causes oxidative damage to lipids and mitochondrial dysfunction. Following the occurrence of lipid peroxidation, a reduction in the membrane fluidity is observed, which might decrease the fusion of sperm and oolemma and, consequently, decrease the fertilization rate (Lord & Aitken, 2013). An alteration of the lipidome, associated with oocyte ageing, was evaluated in a mouse model (Mok et al., 2016). In addition, the above-mentioned study reported that several phospholipid classes significantly decreased in aged oocytes, which suggests the involvement of oxidative stress in lipid plasma membrane composition and, as a result, unfavourable outcomes of oocyte ageing.

Ageing is associated with increases in the levels of endogenous ROS and decreases in antioxidant defences, leading to a wide range of oxidative damage in cell structures, including lipid peroxidation of membranes, enzyme inactivation, protein oxidation and DNA damage (Dean, Gieseg, & Davies, 1993; Headlam & Davies, 2004; Orrenius, Gogvadze, & Zhivotovsky, 2007). Enzymatic antioxidant system can scavenge ROS and therefore, decreases the effect of oxidative stress. The role of oxidative stress in the ageing of several types of cells and tissues has been implicated, but very little is known about how oocyte antioxidant defences change during oocyte ageing.

Fish are good model animals to evaluate oocyte ageing, because they display a vast diversity of reproductive modes, and most produce a high number of oocytes compared to the other animals. Since the demand for assisted reproduction technology is increasing, studies on the process of oocyte ageing would be beneficial to this purpose. Tench *Tinca tinca* L. is a fresh- and brackish-water fish that belongs to the family Cyprinidae and is found throughout Eurasia. This species is endemic in Central & South Europe (Rodríguez et al., 2008) and, recently, is considered the most promising species in freshwater aquaculture of the countries at these area (Kujawa, Kucharczyk, Mamcarz, Żarski, & Targońska, 2011) due to its role at making diversification in the suitable species for the inland aquaculture (Policar & Adamek, 2013). Tench is of high commercial value in several European countries, such as the Czech Republic, Hungary, Italy, Spain and Poland and in other continents (Kucharczyk et al., 2014). According to the conservation status of the IUCN, tench is now cathegorized as a least concern species (http://www.iucn.org/). On the other hand, the hatching success, in the case of this species, is very low and is reported approximately 30% (Flajshans et al., 2007; Kokurewicz, 1981; Linhart & Kvasnička, 1992). The above-mentioned data emphasizes the necessity for studies optimizing tench hatchery techniques, including controlled artificial reproduction for supporting aquaculture section as well as restocking programmes. Different aspects of tench artificial reproduction (Kujawa et al., 2011; Targońska et al., 2012) and gamete management (Mamcarz, Kucharczyk, & Kujawa, 2006) have been studied. However, the knowledge of tench hatchery protocols is still very limited and needs to be improved.

The time period during which unfertilized eggs retain their fertilizing ability after stripping has not yet been accurately studied for tench. The possibility of in vitro egg storage in tench hatcheries may permit synchronous artificial fertilization for different females and thereby facilitate the hatchery management. This approach might also be helpful when completely mature male brood fish are unavailable and be used as well for the shipment of eggs during the successful storage time. This study therefore, was conducted to identify the successful in vitro egg storage duration in tench as well as the probable alteration in the activity of the egg antioxidant enzymes during 10 hr post stripping (HPS). In addition, the levels of fatty acids and lipid class composition, different enzymatic antioxidant agents, and the markers of oxidative damage on lipids and proteins were measured during prolonged in vitro oocyte ageing. The combination of the above-mentioned parameters will provide a considerably broader picture and understanding of the ongoing mechanisms and facilitate the development of strategies to prevent or delay the oocyte ageing process.

2 | MATERIALS AND METHODS

2.1 | Ethics

The experimental procedures were performed according to the ethical rules of the EU-harmonized Animal Welfare Act of the Czech Republic. The unit is licensed (No. 53100/2013-MZE-17214) according to the Czech National Directive (the Law against Animal Cruelty, No. 246/1992).

2.2 | Fish

In the middle of June 2015, brood fish were captured from earthen ponds (18 ± 1°C) of the experimental facilities at the South Bohemian Research Centre of Aquaculture and Biodiversity, Hydrocenoses, Vodnany, Czech Republic. The fish (~150 females and ~200 males) were then transferred to indoor rectangular-shaped tanks (each 5.7 m^3 capacity) supplied with water from a recirculating system and were kept under dim conditions (<20 lx). The females and males were housed separately. The holding temperature was gradually (0.5°C per day) increased to 21°C. After a 2-day period of acclimation, the female brood fish were treated with a muscular injection of Ovopel (mammalian GnRH analogue [D-Ala^6, Pro^9Net-mGnRH] with metoclopramide-dopamine antagonist). The first injection was done at the dosage of 0.1 and the second one was at 1 pellet/kg of the female's body weight, considering 12- hr intervals (Kujawa et al., 2011). One Ovopel pellet contains 18–20 μg mammalian mGnRHa

[D-Ala^6, Pro^{9}NEt] and 8–10 mg metoclopramide (Horváth, Szabó, & Burke, 1997).

The male brood fish were subjected to a single intramuscular injection of the carp pituitary hormone (CPH) suspension in a physiological saline solution (1 mg/kg body weight) (Linhart, Rodina, Kocour, & Gela, 2006), which was administered simultaneously with the first injection in the females. The females were examined for ovulation every 2 hr, starting 12 hr after the second injection by a gentle palpation of the abdomen. At the first examination, the ovulated females were excluded. The inspection was performed 2 hr later again. Six females, which ovulated within 2 hr, weighing between 0.7 and 1 kg, were selected randomly and were used for the experiment. To examine ovulation and to collect the gametes, the fish were anaesthetized with 0.6 ml/L 2-phenoxyethanol to minimize stress and to make them easier to handle (Flajshans et al., 2007).

2.3 | In vitro egg storage in the ovarian fluid

The stripped ova of six females were collected separately and were stored in six-well sterile cell culture plates (each well diameter: 3.5 cm). Inside each well of the culture plates, 7 g of the eggs were stored. Therefore, around 40 g of eggs from each female were stored at each plate. The eggs were stored two layers deep (Komrakova & Holtz, 2009). All the plates were individually covered by their own lids and were kept in the dark at 20°C in the laboratory incubator for 10 hr. The stored ova were fertilized at 0 (immediately after stripping), 2, 4, 6, 8 and 10 HPS.

2.4 | Artificial fertilization

Ten mature males weighing between 0.3 and 0.5 kg were randomly selected for the experiment. Just before the artificial fertilization at each HPS, 1 ml milt from three males were collected separately into tubes with modified Kurokura immobilizing solution 180 (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$ $2H_2O$ and 2.38 mM $NaHCO_3$; Rodina, Cosson, Gela, & Linhart, 2004). To provide a uniform fertilizing ability for all the egg batches, 0.5 ml of the milt from each of the three males pooled. A total volume of 1.5 ml was then mixed gently and stored at 4°C until it was used for fertilization. Sperm motility was assessed for each male separately before egg insemination by observing the sperm under a microscope at a magnification of ×40 using a glass slide without a coverslip (Fauvel, Suquet, & Cosson, 2010). For each fertilization step, a batch of ~250 eggs from each female was gently dispersed into separate small petri dishes and was then fertilized using 0.15 ml of the mixed milt with the addition of 2 ml of the hatchery water using continuous shaking for 3 min.

2.5 | Incubation and examining the embryo viability rates

After fertilization, the eggs were washed 4–5 times with the hatchery water to remove the remnants of milt. The petri dishes were left for 5 min to ensure that the eggs were attached to the surface. Thereafter, the plates were moved and placed separately into small rectangular-shaped incubators (each 4.5 L capacity) with recirculating water at 20 ± 0.5°C and flow rate of 1 L/min through each incubator. After 24 hr of fertilization, when the eggs reached to the gastrula stage, the embryo survival rates were calculated using a stereomicroscope (Nikon SMZ745T, Japan) with respect to the total number of initially fertilized eggs.

2.6 | Egg sampling

Just before fertilization at each HPS, samples of unfertilized eggs from each female were taken separately and were placed into cryotubes. Then, they were immediately frozen in liquid nitrogen and stored at −80°C for the lipid and protein analyses and antioxidant enzymes activity assays.

2.7 | Antioxidant enzymes activity assays

2.7.1 | Chemicals

All chemicals for determination of antioxidant enzyme activity were purchased from Sigma–Aldrich Corporation (Europe). All organic solvents of HPLC grade were purchased from Merck (Darmstadt, Germany).

2.7.2 | Preparation of post-mitochondrial supernatant

Samples of fish eggs (approx. 400 mg) were homogenized using a Tissue Lyser II, Quagen in 0.1 M K-phosphate buffer (pH 7.4). Homogenates were centrifuged at 15,000 *g*, 4°C for 15 min in a Micro 200 R. All steps were carry out on ice. Supernatant was used for determination of protein concentration and all antioxidant enzymatic activities. Colorimetric method based on bicinchoninic acid (BCA) was used to determine total protein concentration (Smith et al., 1985). Purple complex with cuprous ions formed during BCA reaction was measured at 562 nm. Bovine serum albumin was used as a standard. The samples were diluted to a protein content of 10 mg/mL.

2.7.3 | Antioxidant enzyme activity

Catalase (CAT) activity was determined as a decrease in hydrogen peroxide in a 96-well flat-bottomed UV-transparent microtitre plate. Catalase activity was assessed spectrophotometrically at 240 nm following the method of Claiborne (1985). Calculations were made using a molar extinction coefficient of 40/M/cm.

The superoxide dismutase (SOD) activity was determined by the method of Nishikimi, Rao, and Yagi (1972). Superoxide dismutase activity was assessed spectrophotometrically at 420 nm and expressed as the nitro blue tetrazolium (NBT) formation.

Glutathione peroxidase (GPX) activity was measured using the method of Mohandas, Marshall, Duggin, Horvath, and Tiller (1984). Briefly, incubation mixture (0.3 mL) contained post-mitochondrial supernatant (approx. 0.2 mg of protein), K-phosphate buffer 0.05 M (pH 7.0), in EDTA 1 mM, sodium azide 1 mM and glutathione reductase from baker's yeast (7.5 ml from stock containing 1 U/ml), reduced glutathione 4 mM and NADPH 0.8 mM. The reaction was started via the addition of 0.5 mM of hydrogen peroxide. The oxidation of NADPH was recorded at 340 nm. Calculations were made using the molar extinction coefficient 6,220/M/cm.

The glutathione reductase (GR) activity was measured by the method of (Cribb, Leeder, & Spielberg, 1989).

Briefly, incubation mixture (0.25 ml) contained post-mitochondrial supernatant (approx. 0.3 mg of protein), K-phosphate buffer 0.05 M (pH 7.0), NADPH (0.4 mM), oxidized glutathione (0.4 mM) and diethylenetriaminepentaacetic acid (DTPA) (1 mM). Disappearance of NADPH was measured at 340 nm and calculated as NADPH oxidized formation using molar extinction coefficient of 6,220/M/cm.

All assays were performed spectrophotometrically in quadruplicate or threereplicate using a 96-well microplate reader (Tecan infinite M200, Germany). Samples were held on ice; measurements were made at 25°C. Variation in absorbance at each reaction well was linear over time ($R^2 > .8$). Enzyme activity was expressed as units (μmol for CAT, SOD and GR; nmol for GPX) of substrate oxidized per mg of protein per minute (mean ± *SD*).

2.8 | Lipid analysis of the oocyte samples using gas chromatography

The lipids were extracted from the oocytes according to Hara and Radin (1978), as earlier described by Pickova, Dutta, Larsson, and Kiessling (1997) using hexane:isopropanol (3:2) as the extraction solvent. The extracted lipids were dissolved in 1 ml hexane and lipid content was determined gravimetrically by weighing the extract with a microbalance. Fat content was then calculated as percentage of oocyte weight. Fatty acids from the total lipids were methylated with a boron triflouoride-methanol complex (Appelqvist, 1968) and were subsequently analysed by gas chromatography using a BPX 70 column (SGE, Austin, TX, USA), which had a length of 50 m, an id of 0.22 mm and a film thickness of 0.25 μm (Sampels, Turner, Ostrom, & Pickova, 2010). The peaks were identified by comparing their retention times with those of the standard mixture GLC-68D (Nu-Chek Prep, Elysian, MN, USA) and other authentic standards (Nu-Chek Prep, Elysian, MN, USA; Larodan, Sweden). For method validation check Pickova et al. (1997) and Sampels and Pickova (2011).

2.9 | Lipid analysis of the oocyte samples using thin layer chromatography

Automated high performance thin layer chromatography (HPTLC) was used to investigate the general lipid class composition and phospholipid (PhoL) class composition according to Olsen and Henderson (1989). Samples were diluted to 1 μg/μl in hexan and 5 μg of lipids were applied on pre-coated with silica gel TLC plates (20 × 10 cm; Silicagel 60; 0.20 mm layer, Merck, Darmstadt, Germany) with a CAMAG TLC Sampler ATS 4 (Camag Switzerland). Separation of the lipid classes and PhoL was executed with a CAMAG Automatic Developing Chamber 2 (ADC 2) (Camag Switzerland). For the separation of the lipid classes, hexane:diethyl ether:acetic acid (85:15:2; v/v/v) was used as a mobile phase, whereas methyl-acetate:isopropanol:chloroform:methanol:KCl (25:25:25:10:10; v/v/v/v/v) was used for the separation of the PhoL classes. After separation the plates sprayed for staining with a solution of 3% cupric acetate in 8% phosphoric acid and then charred for 20 min at 140°C. Subsequently analysis of the separated lipid classes was done by scanning the plates with a CAMAG TLC Scanner 3 (Camag, Switzerland). The scanning was performed at a speed of 20 mm/s and a data resolution of 100 μm/step, with a slit dimension of 6.00 × 0.45 mm at a wavelength of 350 nm. The identification of the lipid classes was performed by a comparison of the retention factor with an external standard (TLC 18-3 alpha B and 18-5 B, Nu-Chek Prep, Elysian, MN, USA), while the PhoL classes were compared to authentic single standards (phosphatidylinositol, sphingomyelin, phosphatidylserin, lysophosphatidylcholine, phosphatidylcholine, phosphatidic acid, cardiolipin and phosphatidylethanolamine) (Avanti Polar Lipids, Inc., AL, USA). Quantification was done by the measuring the area of each spot. For the data filtering, the mode Savitsky-Golay 7 and manual baseline correction were used. Both lipid classes and PhoL classes were calculated and presented as percentage of total lipids identified.

2.10 | Thiobarbituric acid reactive substances

The thiobarbituric acid reactive substances (TBARS) method was used to evaluate oocyte lipid peroxidation according to Li et al. (2010). After reaction with thiobarbituric acid in darkness for 15–20 hr (overnight) at room temperature (20°C), the reaction complex was detected at a 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 μg/g.

2.11 | Protein oxidation

Protein oxidation was estimated as carbonyls after incubation with 2,4-dinitrophenylhydrazine (DNPH) in 2 N hydrochloric acid following a slightly modified method described by Oliver, Ahn, Moerman, Goldstein, and Stadtman (1987). The Carbonyl concentration was calculated from absorption at 370 nm and a molar extinction coefficient of 21.0 mM^{-1} cm^{-1}. The protein concentration was measured at 280 nm in the same sample and quantified using bovine serum albumin as a standard.

2.12 | 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, the activity of antioxidant enzymes, the fatty acid and lipid class composition, and 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. The differences among females for the examined parameters were calculated based on the results obtained from three separate samples from each female immediately after the egg stripping. Multiple regressions were used considering the fertilization rate as the dependent (variable) value and the Cholesterol and triacylglycerols (TAG) as the independent parameters.

3 | RESULTS

3.1 | Embryo survival rates through ova ageing

The highest embryo survival percentages (35 ± 11%; mean ± *SD*) were obtained for the eggs fertilized immediately after stripping (Figure 1). After 4 hr of in vitro egg storage at 20°C, the embryo survival rates were still 68% of their initial rates at 0 HPS. Thereafter, the values decreased significantly over time and dropped to 5 ± 3.7% for the eggs fertilized at 10 HPS.

3.2 | Effect of in vitro oocyte ageing on the activity of antioxidant enzymes

The activity of CAT was not significantly different between during post-stripping oocyte ageing (Figure 2). However, significantly higher SOD levels were observed in eggs at 6 HPS (Figure 3). Activity of GR and GPX were the highest at 0 HPS. With prolonged in vitro storage of the oocytes GR activity decreased significantly at 8 HPS (Figure 4). The activity of GPX showed a significant decrease after 4 HPS (Figure 5). Tench oocytes stripped at 8 and 10 HPS showed the depleted level of GPX on 14%.

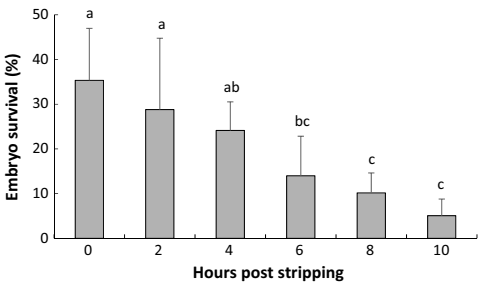

FIGURE 1 Effect of in vitro oocyte ageing at 20°C in tench on the embryo survival rates (mean ± *SD*). The means sharing a common alphabetical symbol do not differ significantly

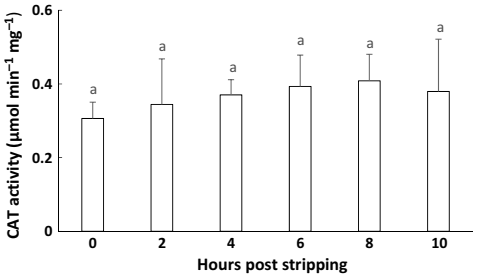

FIGURE 2 Effect of in vitro oocyte ageing at 20°C in tench on the catalase activity. (µmol min^{-1} mg^{-1}; mean ± *SD*)

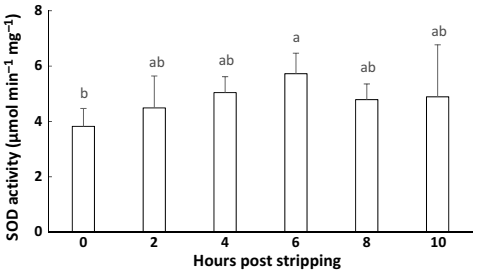

FIGURE 3 Effect of in vitro oocyte ageing at 20°C in tench on the superoxide dismutase activity (µmol $min^{-1}mg^{-1}$; mean ± *SD*)

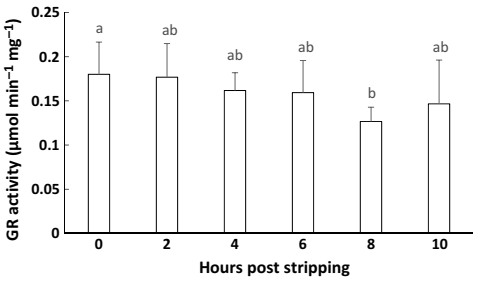

FIGURE 4 Effect of in vitro oocyte ageing at 20°C in tench on the glutathione reductase activity. (µmol $min^{-1}mg^{-1}$; mean ± *SD*)

3.3 | Effect of in vitro oocyte ageing on the lipid and protein oxidation of the oocytes

The level of TBARS, an intracellular stress marker, in the oocytes exhibited no significant changes during post-stripping ageing (Figure 6).

Protein oxidation, measured as carbonyl values, remained constant till 8 HPS. After that the values decreased significantly to 4.9 nmol/mg at 10 HPS (Figure 7).

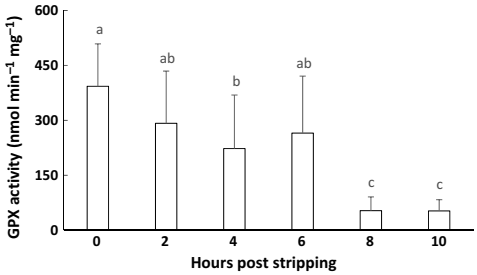

FIGURE 5 Effect of in vitro oocyte ageing at 20°C in tench on the glutathione peroxidase (GPX) activity. (nmol min^{-1} mg^{-1}; mean ± *SD*)

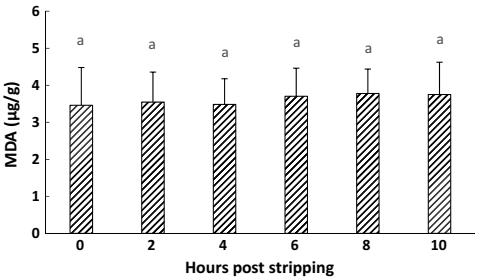

FIGURE 6 Effect of in vitro oocyte ageing at 20°C in tench on TBARs, expressed as malonaldehyde (µg/g; mean ± *SD*)

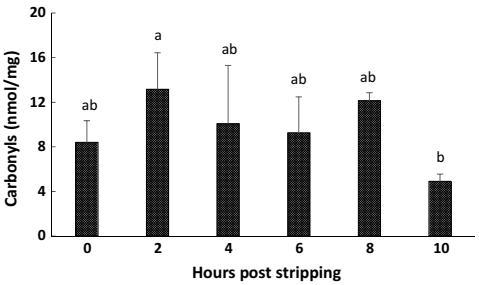

FIGURE 7 Effect of in vitro oocyte ageing at 20°C in tench on carbonyls (nmol/mg; mean ± *SD*)

3.4 | Effect of in vitro oocyte ageing on the fat content and lipid class composition of the oocytes

The fat content of oocytes did not change significantly during oocyte ageing (Table 1). However, the fat content significantly differed among the individual females (R^2 = .7; Table 2).

No significant changes in the main lipid class composition of the oocytes were found during the ageing of the eggs (Table 1). As revealed by TLC, the highest proportions of the identified lipid classes were PhoL, exceeding 60% of total lipids. Cholesterol (Chol) levels varied from 20% to 22%. The amount of TAG was 18%–19.5% of the total lipids identified in the samples. The Chol levels and the content of TAG differed significantly among the individual females (R^2 = .89 for the Chol and R^2 = .53 for the TAG; Table 2).

In addition, the PhoL composition did not show significant differences among the different HPSs (Table 1). The identified PhoL were phosphatidylcholine (PC), at approximately 64%, cardiolipin, at approximately 15% and phosphatidylethanolamine (EA), at approximately 20%. Only the percentage of EA, was significantly different among the individual females (Table 2). The multiple regression results between the fertilization rates and the cholesterol and TAG amounts found a high R square of 0.96.

3.5 | Effect of in vitro oocyte ageing on the fatty acid composition of the oocytes

The main fatty acids (FA) found in the eggs were palmitic acid (16:0), oleic acid (18:1n-9), arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3) and docosahexaenoic acid (22:6n-3; Table 3). During oocyte ageing, no changes in the egg fatty acid composition were found. The ratio of *n*-3/*n*-6 FA found in the samples did not differ significantly over time. The FA composition, however, differed significantly among the individual females (Table 4).

4 | DISCUSSION

The optimal in vitro storage time for the unfertilized eggs varies from a few minutes to a few days among different fish species and depends on the storage temperature (for details see Samarin et al., 2015). In this study, the embryo survival rates for tench eggs that were stored up to 4 hr after stripping at 20°C were not significantly different. This is in accordance with the previously reported results by Flajshans et al. (2007), showing that the fertilization rates of tench eggs mostly decreased after 3–5 hr of in vitro egg storage at 17–24°C.

Biological systems have developed mechanisms to control ROS levels, including enzymatic and non-enzymatic antioxidant agents (Johnson & Nasr-Esfahani, 1994). The activity of antioxidant defense system has not been previously reported in fish during oocyte ageing. In this study, activities of the four chosen enzymes in fish oocytes were assayed with elapsing time after ovulation. The SOD-CAT co-working system is the first defense mechanism of cell against radical toxicity. Their activity implies the presence of a large amount of H_2O_2 in the cell. In this study, SOD activity showed induction at single time point (6 HPS). This response could be involving compensatory response to a stress and adaptation to ROS production.

The detoxification of ROS and hydroperoxides implies the oxidation of GSH to GSSG by GPX. GSSG is then reduced to GSH by GR

TABLE 1 Fat content as percentage of oocyte weight and lipid class and phospholipid class composition as percentage of total detected lipid- and phospholipid classes respectively of tench eggs at different times post stripping

	0 HPS	2 HPS	4 HPS	6 HPS	8 HPS	10 HPS
Fat content (%)	6.08 ± 1.38	5.51 ± 0.70	5.38 ± 1.00	5.60 ± 1.62	6.60 ± 1.96	6.54 ± 2.16
Triacylglycerols (%)	18.1 ± 1.93	18.7 ± 2.65	19.1 ± 2.20	19.6 ± 3.04	19.0 ± 1.97	19.5 ± 1.03
Cholesterol (%)	22.4 ± 1.08	20.9 ± 1.22	21.8 ± 2.80	22.0 ± 1.82	21.7 ± 1.33	21.5 ± 0.57
Phospholipids (%)	59.3 ± 2.57	60.0 ± 3.16	59.1 ± 3.07	58.3 ± 2.77	58.9 ± 2.08	58.7 ± 0.55
Separated phospholipids						
Phosphatidylcholine (%)	63.0 ± 1.85	64.6 ± 1.40	64.0 ± 2.19	64.4 ± 2.87	64.7 ± 3.32	64.7 ± 1.86
Cardiolipin (%)	16.5 ± 1.88	15.5 ± 0.76	15.6 ± 0.93	16.1 ± 2.21	15.0 ± 4.32	15.3 ± 0.37
Phosphatidylethanol- amine (%)	20.5 ± 0.86	19.9 ± 1.60	20.4 ± 1.82	19.5 ± 1.39	20.7 ± 2.73	20.0 ± 1.68

HPS, hour post stripping.
The values did not show significant difference over time.

TABLE 2 Fat content as percentage of total oocyte weight and lipid class and phospholipid class composition as percentage of total detected lipid- and phospholipid classes respectively of tench eggs in individual females (mean ± *SD*)

Lipid classes	Female individual 1	Female individual 2	Female individual 3	Female individual 4	Female individual 5	Female individual 6
Fat content (%)	5.97 ± 0.67^{a}	6.81 ± 2.82^{b}	5.15 ± 1.26^{c}	6.31 ± 1.10^{a}	4.66 ± 1.06^{ac}	6.04 ± 0.88^{ab}
Triacylglycerols (%)	17.6 ± 2.33^{a}	20.7 ± 1.77^{bc}	23.4 ± 2.24^{b}	19.7 ± 1.69^{ac}	17.0 ± 0.28^{a}	17.5 ± 1.51^{a}
Cholesterol (%)	21.3 ± 1.04^{a}	23.1 ± 1.34^{b}	19.5 ± 1.28^{c}	22.1 ± 1.34^{a}	21.7 ± 0.85^{a}	22.4 ± 0.36^{ab}
Phospholipids (%)	61.0 ± 2.64^{a}	55.3 ± 2.58^{b}	55.7 ± 2.82^{b}	58.4 ± 1.80^{ab}	61.2 ± 0.66^{a}	59.8 ± 1.7^{a}
Separated phospholipids						
Phosphatidylcholine (%)	63.9 ± 1.45^{a}	63.0 ± 2.62^{a}	65.6 ± 2.15^{a}	63.3 ± 2.72^{a}	65.1 ± 1.67^{a}	64.9 ± 2.37^{a}
Cardiolipin (%)	15.4 ± 0.46^{a}	15.5 ± 3.98^{a}	16.2 ± 1.09^{a}	16.9 ± 2.02^{a}	14.2 ± 0.74^{a}	15.6 ± 1.47^{a}
Phosphatidylethanol-amine (%)	20.8 ± 1.15^{a}	21.8 ± 2.21^{a}	18.2 ± 1.25^{b}	19.8 ± 1.54^{ab}	20.7 ± 1.10^{a}	19.5 ± 0.93^{ab}

Means with different superscript letters in a row differ significantly.

at the expenses of NADPH, which is recycled by the pentose phosphate pathway. We found a clear time-dependent decreasing of GPX response in fish oocytes. The drastic decline of GPX might be a sign of formation of radical intermediates by oxygen reduction mechanisms. The levels of ROS in in vitro-aged oocytes have also been reported of higher abundance than in fresh oocytes (Tarin, Perez-Albala, & Cano, 2000). The decline of GPX activity indicates a decreased hydrogen peroxide removal (Flohg, 1979), which could subsequently lead to an increased oxidative stress in the oocytes. The decrease in GPX activity could also indicate a failure of the antioxidant system due to ageing. However, this hypothesis would need some future research. To the best of our knowledge, GPX activity has not been evaluated in fish oocytes related to ageing, but Cetica, Pintos, Dalvit, and Beconi (2001) showed a decrease in GPX during the maturation of bovine oocytes. The authors however, did not conclude on the possible reasons for the observed trend. Despite low level of GSSH formed by GPX reaction, GR was significantly decreased at single time point (8 HPS), probably resulting from differences in the availability of NADPH.

In this study, all investigated biomarkers of antioxidant enzyme activity did not show a time-dependent response except of GPX. This could be interpreted as an adaptive response of the detoxification mechanism to possible oxidative stress. Moreover, our results showed that age-associated changes did not lead to oxidative lipid damage in tench oocytes as indicated by MDA levels. The oocytes however, demonstrated significant age-related decrease in protein oxidation. The presence of enzymatic antioxidant activities in oocytes suggests the need to control ROS levels during in vitro storage.

Ageing fish oocytes decreases the fertilization rates as well as the developmental success of the embryo (Samarin et al., 2015). Since an increasing ROS and, consequently, oxidative stress is proposed as the critical factor responsible for the progress of oocyte ageing in higher vertebrates, the status of lipid and protein oxidation with the progress of oocyte ageing was examined. In this study, the levels of TBARs, as indicator of lipid oxidation, showed the upward trend in the more aged oocytes compared to the freshly ovulated ones, even if the increasing trend was not significant. Previously, the level of lipid peroxidation, which is an indicator of the degree of oxidative stress, was shown to increase in in vivo-aged mouse oocytes (Takahashi, Takahashi, Igarashi, Tezuka, & Kurachi, 2003). In human embryos, a positive correlation has been reported between the concentration of hydrogen peroxide and the occurrence of apoptosis (Yang et al., 1998). The upward expression trend of the genes related to the oxidative injury and stress response is identified during oocyte ageing in African catfish *Clarias*

TABLE 3 Fatty acid composition of tench eggs at different times post stripping

Fatty acids	0 HPS	2 HPS	4 HPS	6 HPS	8 HPS	10 HPS
14:0	1.00 ± 0.46	1.08 ± 0.57	0.96 ± 0.47	1.00 ± 0.50	0.98 ± 0.45	0.99 ± 0.60
16:0	29.5 ± 2.05	29.5 ± 1.56	29.2 ± 1.36	29.1 ± 1.30	28.8 ± 1.11	29.2 ± 1.41
16:1	7.83 ± 0.86	7.86 ± 0.70	7.58 ± 1.04	7.72 ± 0.88	7.41 ± 1.12	7.45 ± 0.98
18:0	6.62 ± 0.66	6.59 ± 1.02	6.92 ± 0.75	6.52 ± 0.77	6.95 ± 0.47	7.06 ± 0.48
18:1n-9	15.8 ± 2.53	15.7 ± 2.62	16.5 ± 2.88	16.2 ± 2.68	17.0 ± 2.54	15.6 ± 2.80
18:1n-7	4.85 ± 0.69	4.86 ± 0.65	4.76 ± 0.72	4.70 ± 0.69	4.89 ± 0.72	4.61 ± 0.54
18:2n-6	5.13 ± 1.45	5.12 ± 1.37	5.36 ± 1.45	5.47 ± 1.47	5.43 ± 1.60	4.98 ± 1.70
18:3n-3	1.35 ± 1.00	1.40 ± 1.09	1.29 ± 0.89	1.31 ± 0.93	1.28 ± 0.97	1.43 ± 1.11
20:1	0.24 ± 0.07	0.23 ± 0.07	0.23 ± 0.08	0.22 ± 0.08	0.27 ± 0.03	0.24 ± 0.06
20:2n-6	0.76 ± 0.18	0.76 ± 0.21	0.77 ± 0.14	0.76 ± 0.13	0.82 ± 0.15	0.73 ± 0.16
20:4n-6	4.84 ± 0.85	4.71 ± 0.48	4.82 ± 0.90	4.95 ± 0.56	5.06 ± 0.64	5.23 ± 0.69
20:3n-3	0.34 ± 0.20	0.39 ± 0.27	0.33 ± 0.18	0.31 ± 0.21	0.34 ± 0.17	0.34 ± 0.20
20:5n-3	6.02 ± 2.35	6.08 ± 2.47	5.70 ± 2.35	5.89 ± 2.30	5.14 ± 1.89	6.20 ± 2.72
22:5n-3	2.29 ± 0.82	2.25 ± 0.86	2.13 ± 0.84	2.19 ± 0.90	1.94 ± 0.73	2.33 ± 1.02
22:6n-3	13.5 ± 1.47	13.4 ± 1.38	13.4 ± 1.36	13.7 ± 1.21	13.7 ± 1.18	13.7 ± 1.34
SFA	37.1 ± 2.13	37.16 ± 2.09	37.1 ± 1.51	36.6 ± 1.53	36.7 ± 1.16	37.2 ± 1.37
MUFA	28.7 ± 2.55	28.7 ± 2.57	29.1 ± 2.87	28.9 ± 2.56	29.6 ± 2.34	27.9 ± 2.68
PUFA	34.2 ± 3.34	34.1 ± 3.35	33.8 ± 3.35	34.6 ± 2.91	33.7 ± 3.12	34.9 ± 3.07
n-3	23.4 ± 4.64	23.6 ± 4.78	22.9 ± 4.46	23.4 ± 4.29	22.4 ± 4.43	24.0 ± 4.92
n-6	10.7 ± 1.76	10.6 ± 1.58	11.0 ± 1.60	11.2 ± 1.62	11.3 ± 1.58	10.9 ± 2.10
n-6/*n*-3	2.28 ± 0.78	2.32 ± 0.80	2.17 ± 0.73	2.18 ± 0.73	2.06 ± 0.75	2.32 ± 0.89

HPS, hour post-stripping; MUFA, monounsaturated fatty acids; *n*-3, omega 3 fatty acids; *n*-6, omega 6 fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
Values are percentage of total identified fatty acids (mean ± *SD*) and did not differ significantly over time ($p > .05$).

gariepinus (Samarin et al., 2018). The intra-cytoplasmic level of glutathione, which plays a major role in protecting oocytes from damage by ROS, decreases in aged mouse oocytes (Boerjan & de Boer, 1990).

Carbonyls values were increased at 2 HPS. The increasing carbonyl concentration showed the occurrence of protein oxidation after 2-hr storage time. However at 4, 6 and 8 HPS, the values decreased to the carbonyls values in freshly ovulated oocytes and then decreased significantly at 10 HPS.

The quantitative and qualitative composition of lipids of the tench eggs did not differ significantly during the prolonged in vitro storage of the eggs. This finding suggests that the deleterious effect of ova ageing is not related to the decreased lipid content. Ova ageing may lead to mitochondrial dysfunction and disturbed ATP production as shown in common carp and rainbow trout (Aegerter & Jalabert, 2004; Boulekbache, Bastin, Andriamihaja, Lefebvre, & Joly, 1989).

Decreased fertilization rates with elapsing time after ovulation, in this study, might be at least partially related to the decreased fusion of sperm to oolemma due to the membrane dysfunction as it was shown before by Lord and Aitken (2013). In this study, we did not examine the membrane integrity during oocyte ageing. This topic can be addressed in the future studies. Alterations of lipids are associated with the developmental competence of the eggs (Dunning, Russell, & Robker, 2014; Jungheim et al., 2011). However, in this study, the lipid content and fatty acid composition of the tench eggs did not differ significantly during the prolonged in vitro storage of the eggs. In line with our results, in the eggs of Eurasian perch *Perca fluviatilis*, the fat content was not correlated with the egg fertilizing ability (Zarski et al., 2011). In contrast, Wu et al. (2010) showed that a higher lipid content in rat oocytes negatively affects fertilization rates. However, the fat content and fatty acid composition of the oocytes differed significantly in the individuals chosen for the experiment, indicating differences in the individual metabolisms due to the genetically or nutritionally differences. In this study, the main lipid class composition and PhoL classes were not significantly changed during the in vitro storage of the eggs. In contrast, it was shown earlier, that ageing in rainbow trout eggs was accompanied by an increase in free lipid and a decrease in lipid phosphorus, as a measure for the PhoLs (Craik & Harvey, 1984). In addition, the levels of esterified and non-esterified fatty acids are significantly decreased in aged rainbow trout eggs (Lahnsteiner, 2000). In Caspian brown trout, the level of TAG decreases 30 days post-ovulation (Bahrekazemi et al., 2010).

However, the lipid class composition and PhoL classes significantly differed among the individual females in this study. The Chol levels differed significantly among the individuals. In this study, the Chol levels were lowest in female 3, which also exhibited the lowest

TABLE 4 Fatty acid composition of tench eggs in individual females. Values are percentage of total identified fatty acids (mean ± *SD*)

Fatty acids	Female individual 1	Female individual 2	Female individual 3	Female individual 4	Female individual 5	Female individual 6
14:0	$0.86^{a} \pm 0.06$	$0.66^{b} \pm 0.05$	$1.90^{c} \pm 0.14$	$0.61^{b} \pm 0.00$	$0.71^{b} \pm 0.06$	$1.04^{d} \pm 0.10$
16:0	$30.3^{a} \pm 0.33$	$31.09^{a} \pm 0.78$	$27.62^{b} \pm 0.28$	$28.3^{b} \pm 0.41$	$29.2^{c} \pm 0.67$	$28.5^{b} \pm {}^{c}0.61$
16:1	$8.18^{ac} \pm 0.31$	$8.03^{a} \pm 0.32$	$7.95^{ad} \pm 0.30$	$6.67^{b} \pm 0.08$	$6.28^{b} \pm 0.30$	$8.57^{c} \pm 0.40$
18:0	$7.46^{a} \pm 0.36$	$7.24^{ac} \pm 0.28$	$6.06^{d} \pm 0.38$	$7.39^{e} \pm 0.12$	$6.74^{d} \pm 0.45$	$5.87^{e} \pm 0.32$
18:1n-9	$18.4^{a} \pm 0.26$	$13.7^{b} \pm 0.09$	$12.6^{c} \pm 0.18$	$19.8^{d} \pm 0.35$	$17.6^{e} \pm 0.24$	$16.3^{f} \pm 0.14$
18:1n-7	$4.90^{a} \pm 0.06$	$4.02^{b} \pm 0.05$	$5.20^{c} \pm 0.07$	$4.12^{b} \pm 0.07$	$4.33^{d} \pm 0.06$	$5.79^{e} \pm 0.06$
18:2n-6	$6.34^{a} \pm 0.16$	$4.74^{b} \pm 0.10$	$2.75^{c} \pm 0.09$	$6.59^{d} \pm 0.09$	$6.09^{e} \pm 0.12$	$5.77^{f} \pm 0.11$
18:3n-3	$0.68^{a} \pm 0.02$	$1.13^{b} \pm 0.03$	$3.13^{c} \pm 0.10$	$0.97^{d} \pm 0.03$	$0.88^{e} \pm 0.03$	$0.98^{d} \pm 0.06$
20:1	$0.33^{a} \pm 0.02$	$0.15^{b} \pm 0.04$	$0.20^{bc} \pm 0.02$	$0.27^{c} \pm 0.03$	$0.25^{c} \pm 0.03$	$0.24^{c} \pm 0.05$
20:2n-6	$0.76^{a} \pm 0.02$	$0.50^{b} \pm 0.06$	$0.87^{c} \pm 0.03$	$0.67^{d} \pm 0.02$	$0.79^{a} \pm 0.02$	$0.95^{b} \pm 0.05$
20:4n-6	$5.42^{ad} \pm 0.10$	$4.19^{b} \pm 0.28$	$5.21^{c} \pm 0.18$	$4.80^{a} \pm 0.33$	$5.62^{ab} \pm 0.44$	$4.00^{b} \pm 0.36$
20:3n-3	$0.20^{a} \pm 0.04$	$0.25^{a} \pm 0.05$	$0.71^{c} \pm 0.08$	$0.23^{a} \pm 0.02$	$0.27^{ab} \pm 0.03$	$0.34^{b} \pm 0.03$
20:5n-3	$2.81^{a} \pm 0.12$	$8.82^{b} \pm 0.30$	$8.00^{c} \pm 0.11$	$4.27^{d} \pm 0.15$	$4.82^{e} \pm 0.19$	$6.06^{f} \pm 0.29$
22:5n-3	$1.14^{a} \pm 0.04$	$3.33^{b} \pm 0.12$	$2.79^{c} \pm 0.10$	$1.48^{d} \pm 0.06$	$1.73^{e} \pm 0.09$	$2.51^{f} \pm 0.17$
22:6n-3	$12.3^{a} \pm 0.25$	$12.2^{a} \pm 0.30$	$15.0^{b} \pm 0.16$	$13.9^{c} \pm 0.29$	$14.7^{b} \pm 0.22$	$13.2^{d} \pm 0.38$
SFA	$38.6^{a} \pm 0.47$	$39.0^{a} \pm 0.60$	$35.6^{b} \pm 0.33$	$36.3^{b} \pm 0.53$	$36.7^{c} \pm 0.45$	$35.4^{b} \pm 0.69$
MUFA	$31.8^{a} \pm 0.18$	$25.9^{b} \pm 0.40$	$25.9^{b} \pm 0.11$	$30.8^{c} \pm 0.53$	$28.45^{d} \pm 0.35$	$30.8^{c} \pm 0.39$
PUFA	$29.7^{a} \pm 0.59$	$35.1^{b} \pm 0.98$	$38.5^{c} \pm 0.34$	$32.9^{d} \pm 0.83$	$34.9^{bd} \pm 0.78$	$33.8^{bd} \pm 1.03$
n-3	$17.1^{a} \pm 0.42$	$25.68^{b} \pm 0.68$	$29.6^{c} \pm 0.30$	$20.9^{d} \pm 0.48$	$22.4^{e} \pm 0.44$	$23.1^{e} \pm 0.79$
n-6	$12.5^{a} \pm 0.18$	$9.42^{b} \pm 0.31$	$8.83^{c} \pm 0.10$	$12.1^{a} \pm 0.35$	$12.5^{a} \pm 0.35$	$10.7^{d} \pm 0.27$
n-3/*n*-6	$1.37^{a} \pm 0.02$	$2.73^{b} \pm 0.03$	$3.36^{c} \pm 0.04$	$1.73^{d} \pm 0.01$	$1.79^{e} \pm 0.02$	$2.16^{f} \pm 0.04$

MUFA, monounsaturated fatty acids; *n*-3, omega 3 fatty acids; *n*-6, omega 6 fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids. Different superscript letters in a row are significantly different.

embryo survival rate as well the highest level of TAG. Female 5, with the highest embryo survival rate at 0 HPS, on the other hand, had the lowest percentage of TAG and a quite high level of Chol (22% of the total identified main lipid classes). We, therefore, speculate that low Chol and high TAG levels are associated with low fertilization. In addition, although it was not significant, there was a slight increase in TAG over time, which could confirm a connection between the TAG percentage and the fertilization capacity. Since the fertilization rate of tench eggs is not high even in freshly ovulated eggs, for future studies it might be advisable to select species models with high fertilization rates to reduce the influence of the intrinsic initial low fertilizing ability of eggs.

The PhoL constitute the highest proportion of the various lipid classes in the total lipids of the eggs of cod and many other marine species. Within the PhoL the PC have the highest percentage (Fraser, Gamble, & Sargent, 1988), which is similar in tench egg composition in this study, where PhoL showed to be the major fraction of the total lipids in tench oocytes (58%–60% of total lipids). In the species with a lower content of TAG in the eggs, the PhoL plays an important role as energy source for the embryos in addition to being a structural component of the membranes (Fraser et al., 1988; Tocher, Fraser, Sargent, & Gamble, 1985). However, in this study, the levels of the different PhoL classes in fish eggs did not differ with different fertilization rates. In contrast to our findings, Mansour, Lahnsteiner, Mcniven, Richardson, and Pelletier (2011) evaluated changes in the FA composition in relation to the fertility of the fish eggs and found significant differences between the eggs with high and low fertility rates. More recently Mok et al. (2016) also reported that several PhoL classes, including phosphatidic acid, phosphatidylinositol, phosphatidylserine, and lysophosphatidylserine, significantly decreased both in H_2O_2-treated and aged oocytes.

5 | CONCLUSION

This study demonstrated that tench eggs could be successfully stored in vitro for 4 hr after stripping at 20°C. The in vitro oocyte ageing is associated with altered activity of oocyte antioxidant enzymes, mainly GPX. Neither FA, major lipid class, nor PhoL class composition was altered during oocyte ageing. However, individual females exhibited different egg qualities, showing differences in FA and lipid class composition as well as the percentage of phosphatidylethanolamine. The level of oxidized proteins decreased during oocyte ageing.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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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*, 59–71. https://doi.org/10.1016/j.aquaculture.2003.08.019

Appelqvist, L.-Å. (1968). Rapid methods of lipid extraction and fatty acid methyl ester preparation for seed and leaf tissue with special remarks on preventing the accumulation of lipid contaminants. Royal Swedish Academy of Science (Kungliga Svenska Vetenskapsakademien). *Stockholm*, *28*, 551–570.

Bahrekazemi, M., Soltani, M., Matinfar, A., Abtahi, B., Pusti, I., Mohagheghi Samarin, A., & Mojazi Amiri, B. (2010). Biochemical and histological studies of over-ripened oocyte in the Caspian brown trout (Salmo trutta caspius) to determine biomarkers for egg quality. *Iranian Journal of Fisheries Sciences*, *9*(1), 33–48.

Bobe, J., & Labbe, C. (2010). Egg and sperm quality in fish. *General and Comparative Endocrinology*, *165*, 535–548. https://doi.org/10.1016/j.ygcen.2009.02.011

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*, 155–163. https://doi.org/10.1002/(ISSN)1098-2795

Bonnet, E., Fostier, A., & Bobe, J. (2007). Microarray-based analysis of fish egg quality after natural or controlled ovulation. *BMC Genomics*, *8*, 55. https://doi.org/10.1186/1471-2164-8-55

Boulekbache, H., Bastin, J., Andriamihaja, M., Lefebvre, M., & Joly, C. (1989). Ageing of fish oocytes: Effects on adenylic nucleotides content, energy charge and viability of carp embryo. *Comparative Biochemistry and Physiology*, *93*, 471–476.

Cetica, P. D., Pintos, L. N., Dalvit, G. C., & Beconi, M. T. (2001). Antioxidant enzyme activity and oxidative stress in bovine oocyte in vitro maturation. *IUBMB Life*, *51*, 57–64. https://doi.org/10.1080/15216540152035073

Claiborne, A. (1985). Catalase activity. In R. A. Greenwall (Ed.), *CRC handbook of methods in oxygen radical research* (pp. 283–284). Boca Raton, FL: CRC Press.

Craik, J. C. A., & Harvey, S. M. (1984). Biochemical changes associated with overripening of the eggs of rainbow trout, *Salmo gairdneri*. *Aquaculture*, *37*, 347–357. https://doi.org/10.1016/0044-8486(84)90299-0

Cribb, A. E., Leeder, J. S., & Spielberg, S. P. (1989). Use of a microplate reader in an assay of glutathione-reductase using 5,5'-dithiobis(2-nitrobenzoic acid). *Analytical Biochemistry*, *183*, 195–196. https://doi.org/10.1016/0003-2697(89)90188-7

Dean, R. T., Gieseg, S., & Davies, M. J. (1993). Reactive species and their accumulation on radical-damaged proteins. *Trends in Biochemical Sciences*, *18*, 437–441. https://doi.org/10.1016/0968-0004(93)90145-D

Dunning, K. R., Russell, D. L., & Robker, R. L. (2014). Lipids and oocyte developmental competence: The role of fatty acids and β-oxidation. *Reproduction*, *148*, 15–27. https://doi.org/10.1530/REP-13-0251

Fauvel, C., Suquet, M., & Cosson, J. (2010). Evaluation of fish sperm quality. *Journal of Applied Ichthyology*, *26*, 636–643. https://doi.org/10.1111/j.1439-0426.2010.01529.x

Flajshans, M., Kohlmann, K., & Rab, P. (2007). Autotriploid tench *Tinca tinca* (L.) larvae obtained by fertilization of eggs previously subjected to postovulatory ageing *in vitro* and *in vivo*. *Journal of Fish Biology*, *71*, 868–876. https://doi.org/10.1111/j.1095-8649.2007.01557.x

Flohg, L. (1979). *Glutathione peroxidase: Fact and fiction, Oxygen free radicals and tissue damage* (pp. 95–122). Amsterdam, Netherlands: Excerpta Medica.

Fraser, A. J., Gamble, J. C., & Sargent, J. R. (1988). Changes in lipid content, lipid class composition and fatty acid composition of developing eggs and unfed larvae of cod (*Gadus morhua*). *Marine Biology*, *99*, 307–313. https://doi.org/10.1007/BF02112122

Hara, A., & Radin, N. S. (1978). Lipid extraction of tissues with low toxicity solvent. *Analytical Biochemistry*, *90*, 420–426. https://doi.org/10.1016/0003-2697(78)90046-5

Harman, D. (1988). Free radicals in aging. *Molecular and Cellular Biochemistry*, *84*, 155–161. https://doi.org/10.1007/BF00421050

Headlam, H. A., & Davies, M. J. (2004). Markers of protein oxidation: Different oxidants give rise to variable yields of bound and released carbonyl products. *Free Radical Biology and Medicine*, *36*, 1175–1184. https://doi.org/10.1016/j.freeradbiomed.2004.02.017

Horváth, L., Szabó, T., & Burke, J. (1997). Hatchery testing of GnRH analogue-containing pellets on ovulation in four cyprinid species. *Polskie Archiwum Hydrobiologii*, *44*, 221–226.

Johnson, M. H., & Nasr-Esfahani, M. H. (1994). Radical solutions and culture problems: Could free oxygen-radical s be responsible for the impaired development of preimplantation mammalian embryos *in vitro*? *Bioassays*, *16*, 31–38. https://doi.org/10.1002/(ISSN)1521-1878

Jungheim, E. S., Macones, G. A., Odem, R. R., Patterson, B. W., Lanzendorf, S. E., Ratts, V. S., & Moley, K. H. (2011). Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during *in vitro* fertilization. *Fertility and Sterility*, *95*, 1970–1974. https://doi.org/10.1016/j.fertnstert.2011.01.154

Kokurewicz, B. (1981). Effect of different thermal regimes on reproductive cycles of tench *Tinca tinca* (L.). Part VII. Embryonic development of progeny. *Polskie Archiwum Hydrobiologii*, *28*, 243–256.

Komrakova, M., & Holtz, W. (2009). Factors responsible for successful chilled storage of unfertilized rainbow trout (*Oncorhynchus mykiss*) eggs. *Aquaculture*, *286*, 156–163. https://doi.org/10.1016/j.aquaculture.2008.09.019

Kucharczyk, D., Żarski, D., Targońska, K., Łuczyński, M. J., Szczerbowski, A., Nowosad, J., … Mamcarz, R. (2014). Induced artificial androgenesis in common tench, *Tinca tinca* (L.), using common carp and common bream eggs. *Italian Journal of Animal Science*, *13*, 196–200.

Kujawa, R., Kucharczyk, D., Mamcarz, A., Żarski, D., & Targońska, K. (2011). Artificial spawning of common tench *Tinca tinca* (Linnaeus, 1758), obtained from wild and domestic stocks. *Aquaculture International*, *19*, 513–521. https://doi.org/10.1007/s10499-010-9366-z

Lahnsteiner, F. (2000). Morphological, physiological and biochemical parameters characterizing the overripening of rainbow trout eggs. *Fish Physiology and Biochemistry*, *23*, 107–118. https://doi.org/10.1023/A:1007839023540

Lahnsteiner, F., Urbanyi, B., Horvath, A., & Weismann, T. (2001). Bio-markers for egg quality determination in cyprinid fish. *Aquaculture*, *195*, 331–352. https://doi.org/10.1016/S0044-8486(00)00550-0

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*, 852–858. https://doi.org/10.1095/biolreprod.110.085852

Linhart, O., & Kvasnička, P. (1992). Artificial insemination in tench, *Tinca tinca* L. *Aquaculture Research*, *23*, 183–188.

Linhart, O., Rodina, M., Kocour, M., & Gela, D. (2006). Insemination and gamete management in tench, *Tinca tinca* (L.). *Aquaculture International*, *14*, 61–73. https://doi.org/10.1007/s10499-005-9014-1

Lord, T., & Aitken, R. J. (2013). Oxidative stress and ageing of the post-ovulatory oocyte. *Reproduction*, *146*, 217–227. https://doi.org/10.1530/REP-13-0111

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*, 1–9.

Mamcarz, A., Kucharczyk, D., & Kujawa, R. (2006). Reciprocal hybrids of tench *Tinca tinca* (L.) × bream *Abramis brama* (L.), and tench × carp *Cyprinus carpio* L., and some characteristics of their early development. *Aquaculture International*, *14*, 27–33. https://doi.org/10.1007/s10499-005-9011-4

Mansour, N., Lahnsteiner, F., Mcniven, M. A., Richardson, G. F., & Pelletier, C. S. (2011). Relationship between fertility and fatty acid profile of sperm and eggs in Arctic char, *Salvelinus alpinus*. *Aquaculture*, *318*, 371–378. https://doi.org/10.1016/j.aquaculture.2011.05.023

McEvoy, L. A. (1984). Ovulatory rhythms and overripening of eggs in cultivated turbot *Scophthalmus maximus* (L.). *Journal of Fish Biology*, *24*, 437–448.

Mohandas, J., Marshall, J. J., Duggin, G. G., Horvath, J. S., & Tiller, D. J. (1984). Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney - possible implications in analgesic nephropathy. *Biochemical Pharmacology*, *33*, 1801–1807. https://doi.org/10.1016/0006-2952(84)90353-8

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*, e0148577. https://doi.org/10.1371/journal.pone.0148577

Nishikimi, M., Rao, N. A., & Yagi, K. (1972). The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochemical and Biophysical Research Communications*, *46*, 849–854. https://doi.org/10.1016/S0006-291X(72)80218-3

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

Olsen, R. E., & Henderson, R. J. (1989). The rapid analysis of neutral and polar marine lipids using double-development Hptlc and scanning densitometry. *Journal of Experimental Marine Biology and Ecology*, *129*, 189–197. https://doi.org/10.1016/0022-0981(89)90056-7

Orrenius, S., Gogvadze, V., & Zhivotovsky, B. (2007). Mitochondrial oxidative stress: Implications for cell death. *Annual Review of Pharmacology and Toxicology*, *47*, 143–183. https://doi.org/10.1146/annurev.pharmtox.47.120505.105122

Pickova, J., Dutta, P. C., Larsson, P. O., & Kiessling, A. (1997). Early embryonic cleavage pattern, hatching success, and egg-lipid fatty acid composition: Comparison between two cod (*Gadus morhua*) stocks. *Canadian Journal of Fisheries and Aquatic Sciences*, *54*, 2410–2416.

Policar, T., & Adamek, Z. (2013). Preface. *Aquaculture International*, *21*, 737–738. https://doi.org/10.1007/s10499-012-9620-7

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*, 26–36. https://doi.org/10.1186/1477-7827-2-26

Rodina, M., Cosson, J., Gela, D., & Linhart, O. (2004). Kurokura solution as immobilizing medium for spermatozoa of tench (*Tinca tinca* L.). *Aquaculture International*, *12*, 119–131. https://doi.org/10.1023/B:AQUI.0000017192.75993.e3

Rodríguez, R., Celada, J. D., Sáez-Royuela, M., Carral, J. M., Aguilera, A., Pérez, J. R., & Melendre, P. M. (2008). Egg production of tench (*Tinca tinca* L.) kept in semi-intensive culture conditions. *Knowledge and Management of Aquatic Ecosystems*, *388*, 04. https://doi.org/10.1051/kmae:2008007

Samarin, A. M., Blecha, M., Uzhytchak, M., Bytyutskyy, D., Żarski, 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. https://doi.org/10.1016/j.aquaculture.2015.08.017

Samarin, A. M., Mojazi Amiri, B., 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*, 12309–12314.

Samarin, A. M., Policar, T., & Lahnsteiner, F. (2015). Fish oocyte ageing and its effect on egg quality. *Reviews in Fisheries Science & Aquaculture*, *23*, 302–314. https://doi.org/10.1080/23308249.2015.1053560

Samarin, A. M., Sampels, S., Policar, T., Rodina, M., Hematyar, N., & Samarin, A. M. (2018). mRNA abundance changes during *in vitro* oocyte ageing in African catfish *Clarias gariepinus* (Burchell, 1822). *Aquaculture Research*, *49*, 1037–1045. https://doi.org/10.1111/are.13552

Sampels, S., & Pickova, J. (2011). Comparison of two different methods for the separation of lipid classes and fatty acid methylation in reindeer and fish muscle. *Food Chemistry*, *128*(3), 811–819. https://doi.org/10.1016/j.foodchem.2011.03.089

Sampels, S., Turner, T., Ostrom, A., & Pickova, J. (2010). Effects of -linolenic acid and eicosapentaenoic acid from linseed and algae, respectively, on reindeer (*Rangifer tarandus tarandus* L.) muscle fatty acid composition. *Acta Agriculturae Scandinavica Section a-Animal Science*, *60*, 175–186. https://doi.org/10.1080/09064702.2010.523092

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., ... Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, *150*, 76–85. https://doi.org/10.1016/0003-2697(85)90442-7

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*, 1431–1439.

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*, *66*, 143–152. https://doi.org/10.1002/(ISSN)1098-2795

Targońska, K., Perkowski, T., Żarski, D., Krejszeff, S., Mamcarz, A., Kujawa, R., & Kucharczyk, D. (2012). Method of evaluation of wild common tench, *Tinca tinca* (L.), female suitability for artificial reproduction during the spawning season. *Italian Journal of Animal Science*, *11*, 164–168.

Tarin, J. J., Perez-Albala, S., & Cano, A. (2000). Consequences on offspring of abnormal function in ageing gametes. *Human Reproduction Update*, *6*, 532–549. https://doi.org/10.1093/humupd/6.6.532

Tocher, D. R., Fraser, A. J., Sargent, J. R., & Gamble, J. C. (1985). Lipid class composition during embryonic and early larval development in Atlantic herring (*Clupea harengus* L.). *Lipids*, *20*, 84–89. https://doi.org/10.1007/BF02534213

Wu, L. L. Y., Dunning, K. R., Yang, X., Russell, D. L., Lane, M., Norman, R. J., & Robker, R. L. (2010). High-fat diet causes lipotoxicity responses in cumulus–oocyte complexes and decreased fertilization rates. *Endocrinology*, *151*, 5438–5445. https://doi.org/10.1210/en.2010-0551

Yang, H. W., Hwang, K. J., Kwon, H. C., Kim, H. S., Choi, K. W., & Oh, K. S. (1998). Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Human Reproduction*, *13*, 998–1002. https://doi.org/10.1093/humrep/13.4.998

Zarski, D., Bokor, Z., Kotrik, L., Urbanyi, B., Horvath, A., Targonska, K., … Kucharczyk, K. (2011). A new classification of a preovulatory oocyte maturation stage suitable for the synchronization of ovulation in controlled reproduction of Eurasian perch *Perca fluviatilis* L. *Reproductive Biology*, *11*, 194–209. https://doi.org/10.1016/S1642-431X(12)60066-7

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CHAPTER 4

ALTERATION OF MRNA ABUNDANCE, OXIDATION PRODUCTS AND ANTIOXIDANT ENZYME ACTIVITIES DURING OOCYTE AGEING IN COMMON CARP *CYPRINUS CARPIO*

Samarin, A. M., Samarin, A. M., Østbye, T. K., Ruyter, B., Sampels, S. Burkina, V., Blecha, M., Gela, D., Policar, T. Alteration of mRNA abundance, oxidation products and antioxidant enzyme activities during oocyte ageing in common carp *Cyprinus carpio* . Manuscript

My share on this work was 30%.

ALTERATION OF MRNA ABUNDANCE, OXIDATION PRODUCTS AND ANTIOXIDANT ENZYME ACTIVITIES DURING OOCYTE AGEING IN COMMON CARP *CYPRINUS CARPIO*

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ABSTRACT

Background: Oocyte ageing is the most important factor affecting egg quality of several fish species after ovulation. Oxidative stress has been proposed as the initiator of the oocyte ageing process in other vertebrates. To identify the role of oxidative stress and apoptosis on the progress of oocyte ageing in the common carp *Cyprinus carpio*, changes in the relative mRNA abundance of selected transcripts were examined by real time quantitative PCR. The possible alteration in the oxidation status of the oocytes during ageing was also examined by measuring thiobarbituric acid reactive substances (TBARS) as the marker for secondary lipid oxidation products and carbonyls, showing the extension of protein oxidation. In addition, the activity of antioxidant enzymes during oocyte ageing was evaluated.

Results: Hatching rates were over 65% up to 4-6 hours post-ovulation (HPO) but linearly decreased thereafter, finally dropping to 1.3% at 12-14 HPO. Hatching rates were more than 70% for the eggs stored *in vitro* up to 6 hours post-stripping (HPS) and then decreased to 21.3% at 10 HPS. The results demonstrated no significant changes in the relative mRNA levels of oxidative stress-related genes or genes involved in the cell cycle during the progress of oocyte ageing in common carp. Additionally, the amount of TBARS and carbonyls did not change as time elapsed following ovulation. The apoptosis-related genes however, were significantly altered following the prolonged time interval between ovulation and fertilization

Conclusions: The lack of response of both activities of antioxidant enzymes and oxidation products during oocyte ageing strengthens the conclusion that oxidative stress is unlikely to be a main factor determining the progress of oocyte ageing in common carp. However, an increase in the mRNA abundance of apoptosis-related genes demonstrates that apoptotic pathway might be involved in the progress of oocyte ageing.

Keywords: *Cyprinus carpio, egg quality, mRNA abundance, oocyte ageing, oxidative stress*

BACKGROUND

Fertilization success, embryo quality and later life of the offspring are highly dependent on the integrity of the oocyte, which contains important information for orchestrating embryogenesis [1, 2] and for remodelling parental genomes [3, 4]. Oocyte ageing, which refers to the time period between ovulation and fertilization, has been identified as the most important factor affecting egg quality of several fish species after ovulation [*e.g.*, 5, 6, 7]. During ovulation, mature eggs are released from follicle cells into the ovarian or body cavity while they still are in metaphase of the second meiotic division stage [8]. The eggs remaining there until spawning is stimulated by environmental factors, or they are collected by artificial techniques. Delayed spawning in nature, delayed egg stripping in culture conditions and delayed fertilization (after egg collection) lead to oocyte ageing, and finally, egg over-ripening. During oocyte ageing, major morphological, physiological, biochemical, histological, cellular and molecular changes occur inside the eggs (for details see Samarin et al*.,* 2015a) [9]. These changes deteriorate the quality of ovulated eggs and lead to a limited fertilization rate [*e.g.*, 10, 11], increased larval malformation [*e.g.*, 12, 13, 14] and increased ploidy anomalies [*e.g.*, 15, 16]. The time period during which eggs remain viable after ovulation and stripping varies from a few minutes to a few weeks depending on the fish species and storage temperature [9].

Oocytes are large cells responsible for embryo development by providing the embryo with transcripts and proteins until the onset of zygotic transcription. Therefore, the dependence of the early stages of embryogenesis on maternal mRNAs and proteins is not surprising [17]. The effect of fish oocyte ageing on several aspects of egg quality has been studied. However, until now, there has been only poor understanding about the processes and underlying mechanism of oocyte ageing in fish, as well as other vertebrates. There are few studies analysing the genomics and transcriptomics of egg quality associated with the oocyte ageing in fish. Different quantities of mRNAs between over-ripened and freshly ovulated rainbow trout *Oncorhynchus mykiss* eggs have been reported [18, 19, 20]. In Atlantic halibut *Hippoglossus hippoglossus*, poor hatching success was correlated with low transcript levels of specific genes [21]. In rainbow trout eggs, ova ageing resulted in the downregulated expression of specific microRNAs and their target genes mainly involved in cell death and signal transduction, stress response and DNA damage, RNA degradation, and energy and transcription regulation [22].

Studies on other vertebrates have proposed the involvement of oxidative stress as the initiating factor on the progress of oocyte ageing [*e.g.*, 23, 24, 25]. These studies report that oxidative stress can, in turn, trigger many cascades affecting oocyte quality, such as mitochondrial dysfunction, DNA damage, perturbed Ca^{2+} homeostasis and lipid peroxidation. In general, ageing is associated with increases in levels of endogenous reactive oxygen species (ROS) and decreases in antioxidant defences; leading to a wide range of oxidative damage in cell structures, including lipid peroxidation of membranes, enzyme inactivation, protein oxidation, and DNA damage [26, 27, 28]. Alteration of the lipidome, associated with oocyte ageing, was evaluated in a mouse model [29]. The latter study reported that several phospholipid classes were significantly decreased in aged oocytes, which suggests the involvement of oxidative damage in lipid plasma membrane composition and, as a result, unfavourable outcomes of oocyte ageing. The enzymatic antioxidant system can scavenge ROS and therefore decrease the effect of oxidative stress. Very little is known about how oocyte antioxidant defences change during oocyte ageing. Additionally, a decline in critical cell cycle factors [30] and impaired mitochondrial function [*e.g.*, 31, 32] have been shown be related to the deleterious effects of oocyte ageing.

Fish are good model animals to evaluate oocyte ageing because they display a vast diversity of reproductive modes, and most species produce a high number of oocytes compared with that of other animals. Due to some ethical concerns and the intrinsic nature of other vertebrates' oocytes, it is difficult to study the oocyte ageing process. Furthermore, as the demand for assisted reproduction technology is increasing and very few studies in this field are available, a comparative study on the process of oocyte ageing in fish might be beneficial for research on other vertebrates. The present study examines some cellular and molecular changes associated with oocyte ageing in the common carp *Cyprinus carpio*, focusing on the possible role of oxidative stress on the progress of the time-dependent oocyte over-ripening process. The evaluation was done at the levels of transcriptome and antioxidant enzyme assays, lipid and protein oxidation status as well as the egg phenotype and functional changes during the oocyte ageing. The common carp was selected for the experiment because the ovulation of the eggs occurs simultaneously in each individual female and because our previous experience with the oocyte ageing in this species was satisfactory regarding practical approaches [11]. Genes involved in oxidative damage and stress response, mitochondrial function, fertilization, embryo development, transcriptional regulation and cell cycling as well as the ones related to the apoptosis were screened for their mRNA abundance during egg over-ripening. We also investigate the alteration in oxidation status of oocytes during post-ovulatory ageing by measuring thiobarbituric acid reactive substances **(**TBARS) as a marker of lipid oxidation, and carbonyls, which show the extent of protein oxidation. In addition, the role of oxidative stress in the progress of oocyte ageing was assessed by evaluation the activity of antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase GR, were examined during oocyte ageing. The combination of the aforementioned parameters will give a broad picture and understanding of the ongoing mechanisms in the oocyte ageing process. Identifying molecular mechanisms involved in the decline of oocyte quality with the progress of oocyte ageing could have important implications for aspects of basic research and practically applications for aquaculture purposes to prevent or delay oocyte ageing.

METHODS

Ethics

The experimental procedures were performed according to the ethical rules of the EU-harmonized Animal Welfare Act of the Czech Republic. The unit is licensed (No. 53100/2013-MZE-17214) according to the Czech National Directive (the Law against Animal Cruelty, No. 246/1992).

Fish

Brood fish were captured from earthen ponds when the average daily water temperature reached 16 °C during the breeding season. The fish were then transferred to indoor rectangular-shaped tanks (each 5.7 m^3 capacity) supplied with water from a recirculating system and maintained under dim conditions (<20 lx), with females and males housed separately. The holding temperature was gradually increased to 20 °C over time.

Egg storage in the ovarian fluid and gamete collection

After a 3-day period of acclimation, female brood fish were treated with an intramuscular injection of carp pituitary homogenate CPH (0.3 mg kg^{-1} body weight). This step was followed by a second injection with CPH (3.5 mg kg^{-1} body weight) 12 hours later. Male brood fish (4.5 ± 0.4 kg body weight, mean ± SEM) were subjected to a single intramuscular injection of CPH (2 mg kg^{-1} body weight) that was given simultaneously with the second injection of the females. These procedures were performed according to Horvath and Tamas, 1985 [33]. The females were examined for ovulation every 2 hours, starting 10 hours after the second injection. To examine ovulation and to collect gametes, brood fish were anaesthetized with a 0.03 ml/L clove oil water bath to minimize stress and to make them easy to handle. Six females that ovulated within 2 hours (weighing 4.2 ± 0.2 kg body weight), were selected randomly, marked with coloured tags and used for the experiment. Almost half of the eggs were stripped from each female and used for the *in vitro* egg storage experiment. The other half of the eggs was retained inside the fish bodies to be used for the *in vivo* egg storage experiment. These two experiments were performed as described below.

In vivo oocyte ageing

In total, seven batches of eggs from each of the six females were separately stripped within 2- hour intervals and then fertilized during the experimental period of 14 hours after ovulation. An egg batch of 7 g was stripped individually from each female and fertilized immediately (0–2 hours post-ovulation, HPO). The rest of the eggs were left inside the ovarian cavity of each female for the next fertilization time. Thus, eggs were retained inside the fish body before fertilization for 0-2, 2-4, 4-6, 6-8, 8-10, 10-12 and 12-14 HPO.

In vitro oocyte ageing

Seven batches of 7 g egg aliquots were collected from each of the six females and stored in sterile six-well cell culture plates (each well diameter: 3.5 cm). The eggs were stored two layers deep [34] in the ovarian fluid, and no solution, artificial media or extender was added. All plates were individually covered by their own lids and stored in the dark at 20 °C in the laboratory incubator for 10 hours. To provide the humidified atmosphere, a few plates were filled with water and placed into the storage chambers [34, 35]. Stored ova were fertilized at 0 (immediately after stripping), 2, 4, 6, 8, 10 and 12 hours post-stripping, HPS.

Artificial fertilization and egg development

For each sampling time, egg quality was evaluated by measurement of the eyeing, eyed-egg mortality, hatching and larval malformation rates. Artificial fertilization, incubation and examination of egg developmental success were performed according to Samarin et al., 2015a [11].

RNA isolation and reverse transcription

At all HPOs and HPSs, 1 g of the unfertilized eggs was placed in cryotubes, frozen in liquid nitrogen and then stored at -80 °C freezer until RNA isolation. Samples were collected in three replicates for each fertilization time.

To prepare total RNA, an equal weight of 20 eggs was taken from each tube and treated

with TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) in Precellys24 homogenizer (Bertin Instruments), 5500 rpm for 2×20 sec with a 5 sec interval. RNA was isolated using the PureLink RNA Purification Kit (Invitrogen) according to the manufacturer's instructions. During RNA isolation, samples were depleted of genomic DNA using a PureLink DNase Kit (Invitrogen) according to the manufacturer's protocol. The RNA concentration and RNA purity were assessed using a NanoDrop Spectrophotometer (ND-1000, Thermo Scientific).

A concentration of 1000 ng RNA was used to generate cDNA using TaqMan reverse transcription Reagents (Life Technologies). Random hexamers were used to prime the reaction. Reverse transcription was performed at 25 °C for 10 min, at 48 °C for 1 hour and finally 95 °C for 5 min. Control reactions were run without TaqMan reverse transcriptase and used as negative controls in the real-time PCR study.

qRT-PCR analysis

Nucleotide sequences corresponding to common carp mRNA (NCBI) were used for primer design (by Primer3) [36, 37]. Transcript levels of 16 genes, *hsp70*, *cox1*, *sod, gpx1*, *cyclinA, cyclinB, jnkA*, *jnkB*, *caspase3A*, *caspase9*, *bax*, *bcl2, cathepsinB, cathepsinZ*, *vasa* and *igf2*, were determined by real time qPCR in duplicate using the LightCycler 480 (Roche Applied Science, Germany). The reaction mix for qPCR consisted of 4 µl diluted (1:10) cDNA, 1 µl forward and reverse primer (final concentration of 500 nM, Table 1), and 5 µl SYBR Green-I Master (Roche). All primers were provided by Invitrogen. A standard curve was included for each primer pair to evaluate primer efficiency. All samples were analysed in parallel, and a non-template control with water substituted for cDNA was run for each primer pair. The qPCR reaction was run under the following conditions: preincubation at 95 °C for 5 minutes, amplification for 45 cycles at 95 °C for 15 seconds and 60 °C for 1 minutes, melting curve at 95 °C for 5 seconds and 65 °C for 1 minutes, cooling at 40 °C for 10 seconds.

The comparative CT method was used for relative quantification of target gene expression levels. To normalize relative gene expressions, three reference genes, glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), 18S ribosomal RNA (*18s*) and beta-actin (*b-actin*), were tested. The mRNA abundance of *gapdh* proved to be highly stable in common carp eggs collected at different time points after ovulation, and it did not show any significant change in Ct values in the eggs at different HPOs and HPSs. Therefore, *gapdh* proved the most stable and was used as the reference gene. Relative expression was then calculated according to the equation $2^{-\Delta Ct}$. Sequences of primers used are listed in Table 1.

Table 1. *qRT-PCR primer sequences.*

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Genbank accession no.
hsp 70	GAGAGGCTGATTGGAGATGC	ACTGCACAACTGGGTCATCA	HQ259767
cytochrome c oxidase I	TCCACGGAGGATCCATTAAA	GGATAGGACGATCCCTGTGA	HQ235998
vasa	AGGCCAGGAAGTTTGCCTAT	TGCAGCCCTTTAACACCTCT	KP661178
Caspase3a	TGATGGCAAAGTATGGCAAA	ATCAAAGACTGGCTGGTTGG	KF055462
Caspase9	CCTGTGGAGGAGGTGAGAAG	ATGGGAATAGCGTCCATCTG	KC676314
18s ribosomal RNA	AGTTCCGACCGTAAACGATG	AGACTCGTGGTTTCCCACAC	JQ619778
beta-actin gene	GCAAAGGTTTTGTGCTCCAT	CATGGATACCGCAAGATTCC	JQ619775
Bcl2	GGGATGCCTTTGTGGAGTTA	TCACTCCTGCCAAGCCTAGT	EU490408
Bcl2 associated X protein ***(Bax)***	GGAGATGAGCTGGATGGAAA	AAGATCTCTCGGGCCACTTT	KJ174685
glutathione peroxidase (Gpx1)	GGAGAAGCTGGAAGTGAACG	TCACCCATCAAGGACACAGA	GQ376155
gapdh	GTGATGCAGGGGCTCAGTAT	CTCTCTTGGCACCACCCTTA	AJ870982
IGF2	TGCAAAACCCATGAAGTCTG	AAGAGGCCCTCCTGAGATGT	KP663718
cyclin A	TGCATGTCTGTCCTGAGAGG	TCCACTTCCGGAGGATACAC	EU380205
cathepsin Z	GAGAGAAAGGCTGGCTCAGA	GGGTCTCCGTACATGCAGTT	AY949988
cathepsin B	AAAGGACCCAGACTCCCTGT	TTTAAGAGTGGGGCAGTTGG	AB215097
Mn/ SOD	TTATGCAGCTTCACCACAGC	ACATCACCCTTAGCCAGTGC	JF411603
cyclin B	CCAGAAAAGCAGCTGTAGCC	TCTTCCTCAAAGCCTGTCGT	EU293852
JNKa	TCGATGAGAGGGAACACACA	GACCTCGAATGACACCGTTT	JN542470
JNKb	CCAACCTCTGCCAAGTCATT	CCGAGTGGAGGTGTTTTGTT	AB001744

ANTIOXIDANT ENZYME ACTIVITY ASSAYS

Preparation of post-mitochondrial supernatant

Samples of fish eggs (approx. 400 mg) were homogenized using a Tissue Lyser II (Qiagen) in 0.1 M K-phosphate buffer (pH 7.4). Homogenates were centrifuged at 15,000 g, 4 °C for 15 min in a Micro 200 R. Supernatants were removed, stored at 0–4 °C and used for protein determination and enzyme assays. Protein levels were measured spectrophotometrically using bovine serum albumin as a standard [38]. The samples were diluted to a protein content of 10 mg/mL.

Antioxidant enzyme activity

Catalase (CAT) activity was quantified as a decrease in hydrogen peroxide in a 96-well flat-bottomed UV-transparent microtitre plate. CAT activity was assessed spectrophotometrically at 240 nm and was performed following the method of Claiborne, 1985 [39]. Calculations were made using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

The superoxide dismutase (SOD) activity was determined by the method of Nishikimi et al., 1972 [40]. Superoxide dismutase activity was assessed spectrophotometrically at 420 nm and expressed as the nitro blue tetrazolium formation per milligram of protein per minute.

Glutathione peroxidase (GPX) activity was measured using the method of Mohandas et al., 1984 [41]. Briefly, incubation mixture (0.3 mL) contained post-mitochondrial supernatant

(approx. 0.2 mg of protein), K-phosphate buffer 0.05 M (pH 7.0), in EDTA 1 mM, sodium azide 1 mM and glutathione reductase from baker's yeast (7.5 ml from stock containing 1 U/ml), reduced glutathione 4 mM and NADPH 0.8 mM. The reaction was started via the addition of 0.5 mM of hydrogen peroxide. The oxidation of NADPH was recorded at 340 nm. Calculations were made using the molar extinction coefficient 6220 M^{-1} cm^{-1}.

The glutathione reductase (GR) activity was measured by the method of Cribb et al., 1989 [42]. Briefly, incubation mixture (0.25 mL) contained post-mitochondrial supernatant (approx. 0.3 mg of protein), K-phosphate buffer 0.05 M (pH 7.0), NADPH (0.4 mM), oxidized glutathione (0.4 mM) and diethylenetriaminepentaacetic acid (DTPA) (1 mM). Disappearance of NADPH was measured at 340 nm and calculated as NADPH oxidized formation using molar extinction coefficient of 6220 M^{-1} cm^{-1}.

All assays were performed spectrophotometrically in quadruplicate or triplicate using a 96-well microplate reader (Tecan Infinite M200, Germany). Samples were held on ice; measurements were made at 25 °C. Variations in absorbance at each reaction well were linear over time (R^2>0.8).

Thiobarbituric acid reactive substances

The thiobarbituric acid reactive substances (TBARS) method was used to evaluate oocyte lipid peroxidation according to the method described by Li et al., 2010 [43]. After reaction with thiobarbituric acid in darkness for 15-20 hours (overnight) at room temperature (20 °C), the reaction complex was detected at a wavelength of 530 nm against the sample blank using a UV-visual plate reader (AF 2200; Austria). The results were expressed as equivalents to malonaldehyde (MDA) in μg/g.

Protein oxidation

Protein oxidation was estimated as carbonyls 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 [44]. Carbonyl concentration was analysed as DNPH calculated on the basis of absorption of 21.0 mM^{-1} cm^{-1} at 370 nm for aliphatic hydrazones. The protein concentration was measured at 280 nm in the same sample and quantified using bovine serum albumin as a standard.

STATISTICAL ANALYSIS

The normality of the data was ascertained using SPSS software version 18. Differences between the means of the groups for each measured egg quality parameter (eyeing, hatching, eyed-egg mortality and larval malformation rates) as well as each individual gene, lipid and protein oxidation levels and antioxidant enzyme activity were evaluated using an ANOVA followed by Duncan's multiple range test. $P < 0.05$ was considered to be significant.

RESULTS

In vivo oocyte ageing

Egg viability

The eyeing and hatching values remained almost constant, approximately 83% and 75%, respectively, for the eggs fertilized up to 4 HPO (Fig. 1). Although not significantly different, the eyeing and hatching rates increased to 89% and 82%, respectively, at 2-4 HPO. Thereafter, the values decreased linearly over time and dropped to 6.3% for the eyeing rates and 1.3% for the hatching rates at 12-14 HPO. Eyed-egg mortality and larval malformation rates were not significantly different for the eggs fertilized up to 6 hours after ovulation. The highest eyed-egg mortality and larval malformation were obtained at 12-14 HPO, 80.9 ± 5.1% and 62.5 ± 11.8%, respectively.

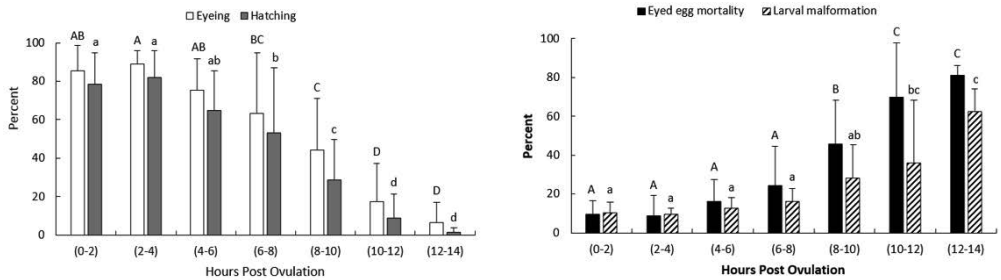

Fig. 1. *Effects of in vivo oocyte ageing on the eyeing, hatching, eyed-egg mortality and larval malformation rates in common carp (mean ± SD). Means sharing a common alphabetical symbol do not differ significantly.*

Relative abundance of mRNA during *in vivo* ova ageing

The mRNA levels of 16 selected transcripts were quantified in freshly ovulated eggs and in aged eggs at different HPOs using *gapdh* as the reference gene. Genes related to the oxidative injury and stress response (*hsp70*, *sod* and *gpx1*) did not show any significant changes in their mRNA abundance during *in vivo* oocyte ageing (Fig. 3). The increased level of *cox1*, however, was observed at 10-12 HPO. Among the genes related to the cell cycle, the mRNA levels of *cyclinA* and *jnkA* increased during oocyte ageing, while *cyclinB* and *jnkB* exhibited constant levels. Relative mRNA abundance of the apoptosis related genes (*caspase3A*, *caspase9* and bax) increased during oocyte ageing while the relative abundance of *bcl2, cathepsinB* and *cathepsinZ* remained constant during this period. Both *vasa* and *igf2* showed a constant levels during post-ovulatory oocyte ageing.

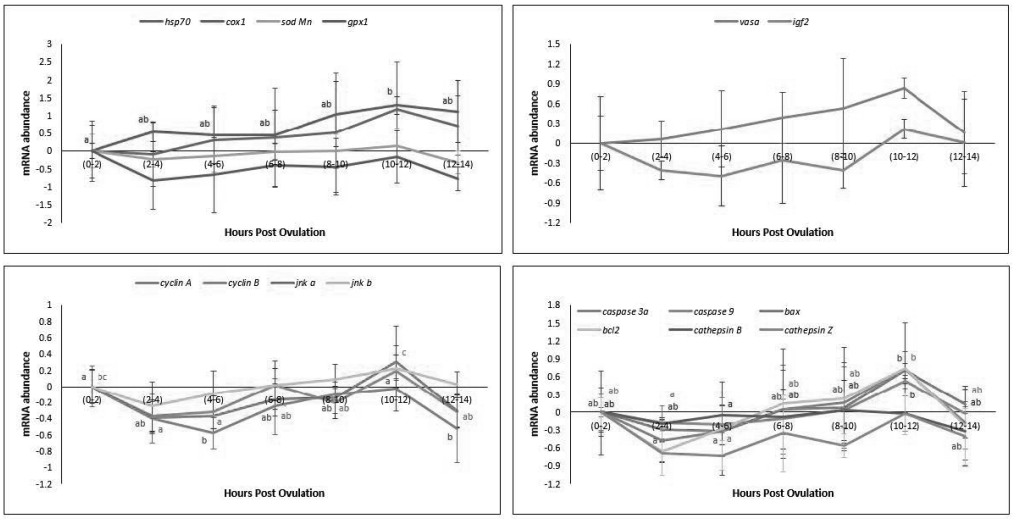

Fig. 3. *Effect of in vivo oocyte ageing on the mRNA levels of the selected transcripts in common carp (mean ± SD).*

Effect of *in vivo* oocyte ageing on the activity of antioxidant enzymes

The activity of antioxidant enzymes, CAT, SOD and GR remained stable with prolonged *in vivo* storage of the oocytes (Fig. 7). The activity of GPX however, increased significantly up to 6-8 HPO.

Effect of *in vivo* oocyte ageing on the lipid and protein oxidation of the oocytes

The level of TBARS, an intracellular stress marker, in the oocytes exhibited no significant changes during post-ovulatory ageing, remaining at approximately 1.5 MDA (μg g^{-1}) (Fig. 5). Protein oxidation, measured as carbonyl values, also did not show any significant changes through 12-14 HPO (Fig. 6).

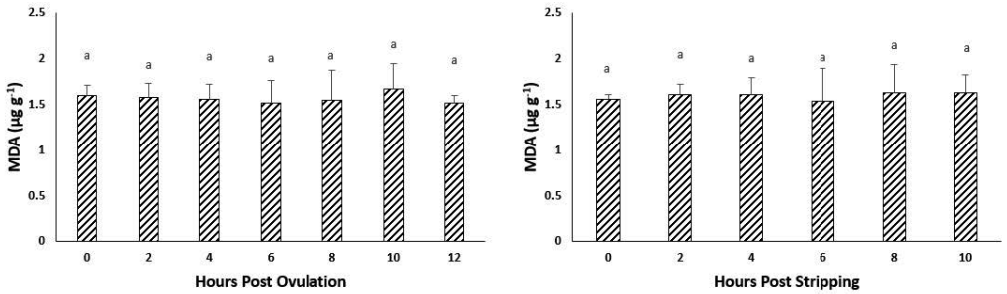

Fig. 5. *Effects of in vivo and in vitro oocyte ageing in common carp on TBARS, expressed as malonaldehyde (MDA) (μg g^{-1}) (mean ± SD).*

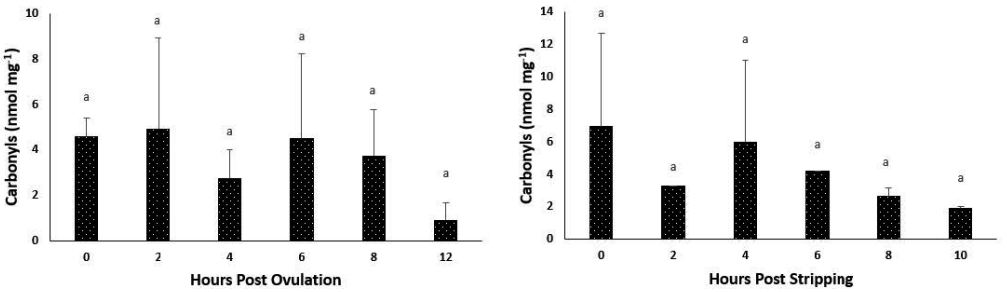

Fig. 6. *Effects of in vivo and in vitro oocyte ageing in common carp on carbonyls (nmol mg^{-1}) (mean ± SD).*

In vitro oocyte ageing

Egg viability

The eyeing and hatching rates were almost constant, approximately 91% and 86%, respectively, up to 6 HPS (Fig. 2). After 10 HPS the eyeing and hatching rates were measured to be 40 ± 5.1% and 21.3 ± 9.7%, respectively. The eyed-egg mortality and larval malformation rates also did not show any marked increase during 6 hours of the *in vitro* egg storage. After 6 HPS, the eyed-egg mortality and larval malformations increased significantly over time so that at 10 HPS, 46% of the eyed-eggs died, and 56% of the hatched larvae were malformed.

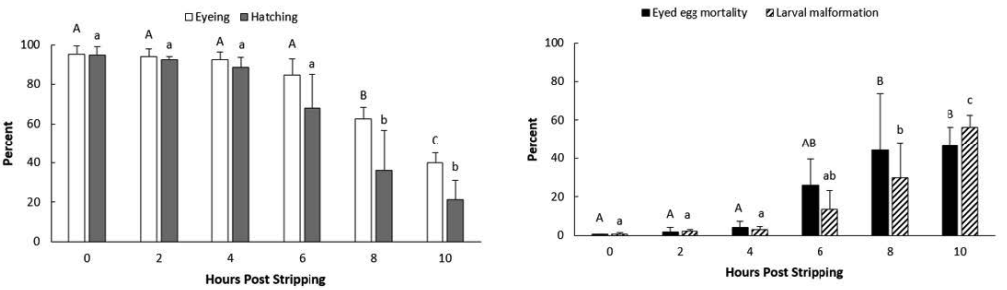

Fig. 2. *Effects of in vitro oocyte ageing on the eyeing, hatching, eyed-egg mortality and larval malformation rates in common carp (mean ± SD).*

Relative abundance of mRNA during *in vitro* ova ageing

The mRNA profiles of the same selected genes were examined during 10 HPS. Except for *jnkB*, none of the examined genes showed any significant changes in their mRNA abundance during *in vitro* oocyte ageing for 10 hours after the stripping (Fig. 4).

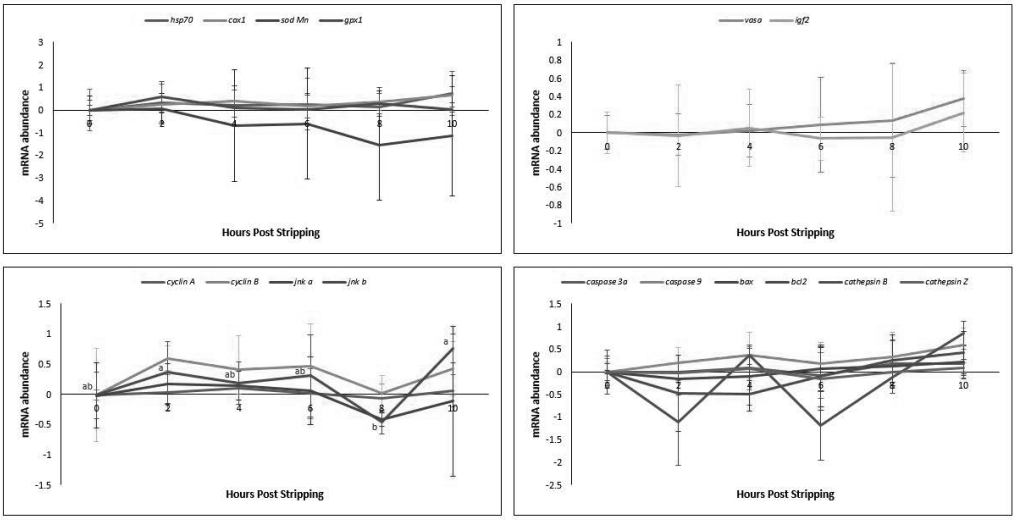

Fig. 4. *Effect of in vitro oocyte ageing on the mRNA levels of the selected transcripts in common carp (mean ± SD).*

Effect of *in vitro* oocyte ageing on the activity of antioxidant enzymes

The CAT, SOD, GR and GPX activities did not show any significant changes through post-stripping oocyte ageing (Fig. 8).

Effect of *in vitro* oocyte ageing on the lipid and protein oxidation of the oocytes

The level of TBARS did not show any significant changes during post-stripping oocyte ageing (Fig. 5). Similarly, carbonyl values, also stayed constant through 10 HPS (Fig. 6).

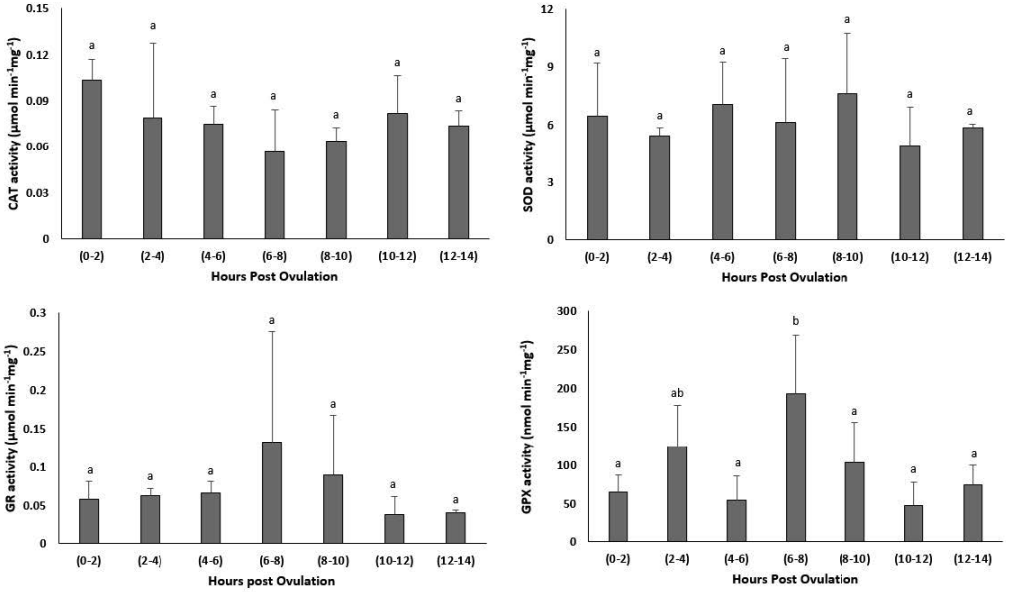

Fig. 7. *Effects of in vivo oocyte ageing in common carp on the activities of catalase (CAT) (µmol/min/mg), superoxide dismutase (SOD) (µmol/min/mg), glutathione reductase (GR) (µmol/min/mg) and glutathione peroxidase (GPX) (µmol/min/mg) (mean ± SD).*

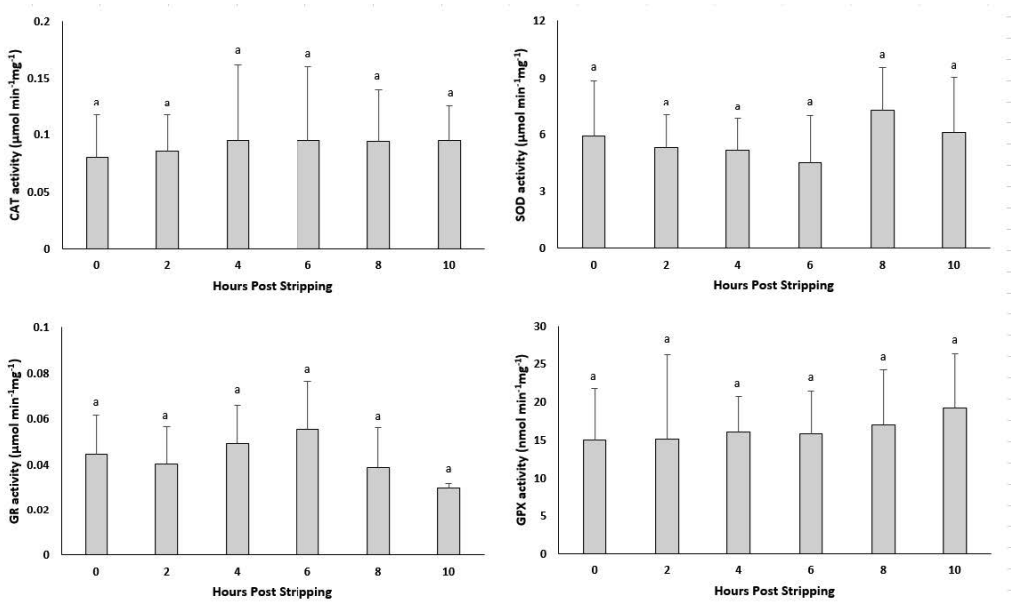

Fig. 8. *Effects of in vitro oocyte ageing in common carp on the activities of catalase (CAT) (µmol/min/mg), superoxide dismutase (SOD) (µmol/min/mg), glutathione reductase (GR) (µmol/min/mg) and glutathione peroxidase (GPX) (µmol/min/mg) (mean ± SD).*

DISCUSSION

With elapsing time after ovulation, oocytes experience changes that negatively affect the egg quality and successive developmental stages. In the present study, the egg quality remained constant when they were stored up to 4-6 hours *in vivo* and 6 hours *in vitro* after ovulation at 20 °C. The observed decreasing trend for the eyeing and hatching rates and the increasing trend for the eyed-egg mortality and larval malformation percentages after ovulation is in accordance with the previously reported experiments on the other fish species [*e.g.*, 10, 16, 18]. However, the successful egg storage time differs from a few minutes to a few weeks and highly depends on the fish species and the storage temperature [9].

It is currently undetermined as to whether a single event acts to trigger a cascade of other factors or if several biochemical and functional changes occur separately to create an aged oocyte [45]. Most authors believe that the onset of ageing in oocytes is associated with an increase in ROS, consequently leading to an increase in oxidative stress [e.g., 23, 24, 25, 45]. The important consequences of oxidative stress in the oocytes are ROS-induced mitochondrial dysfunction, lipid alterations and DNA fragmentation, followed by decreasing ATP production and apoptosis. Esponda and Diaz, 2006 [46] analysed the presence of *hsp70* mRNA and protein in samples collected from 20 HPO aged mice oocytes and found that Hsp70 protein is not present in freshly ovulated oocytes; however, this protein appears in the cytoplasm of aged oocytes. They also reported increased *hsp70* mRNA levels in the aged oocytes. The intracytoplasmic level of glutathione, which has a major role in protecting oocytes from damage by ROS, decreased in aged mouse oocytes [47]. Additionally, the level of lipid peroxidation, which is an indicator of the degree of oxidative stress, increased in the *in vivo*-aged mouse oocytes [24]. In human embryos, a positive correlation has been reported between the concentration of hydrogen peroxide and the occurrence of apoptosis [48], indicating the initiating role of hydrogen peroxide on ageing. Hence, we examined the idea proposed for other vertebrates of whether oxidative stress affects the progress of oocyte ageing. Our results demonstrated no significant changes in the mRNA levels of oxidative stress-related genes or genes involved in the cell cycle during the progress of oocyte ageing in the common carp, with the exception of *cox1*. Additionally, as time elapsed following ovulation, the amount of TBARs (which is the main indicator of lipid peroxidation) and the amount of carbonyls (which show the extension of protein oxidation) did not change in the oocytes, indicating no increase in oxidative stress. Our experiments regarding evaluation the activity of antioxidant enzymes during oocyte ageing have also confirmed that oxidative stress is not likely the main initiator in the progress of oocyte ageing. The enzymatic antioxidant system can scavenge ROS and therefore decrease the effect of oxidative stress. If oxidative stress would be the initiator of deleterious effects during post-ovulatory oocyte ageing, then an alteration in antioxidant enzyme activity and oxidation markers should occur following ovulation. Our results indicated no significant changes in the activity of CAT, SOD and GPX during post-ovulatory ageing of common carp oocytes. The up-regulation of *cox* was also reported in the mouse oocyte with maternal ageing, i.e., the ovarian ageing [32]. On the other hand, *cox1*, located in the mitochondrial membrane, is the last enzyme in the respiratory electron transport chain. Therefore, any changes in the mRNA expression pattern of *cox1* might affect ATP synthesis and promote mitochondrial disfunction. Hamatani et al., 2004 [32] observed a decrease in the transcription levels of ATP-related genes in mouse oocytes during maternal ageing.

The relative mRNA levels of *vasa* in our study showed an upward trend during both *in vivo* and *in vitro* oocyte ageing, however, did not differ significantly. This result is in consistent with the results obtained in another recent experiment by our research group indicating that levels of *vasa* mRNA increases during *in vitro* oocyte ageing in African catfish *Clarias*

gariepinus [49]. *Vasa* is a gene involved in the development of primordial germ cells (PGCs), and its activity is required for both differentiation of the germ cells into gametes [50] and the functionality of germ cells [51]. Loss of *vasa* function in the mouse affects differentiation of the male germ cells, resulting in male sterility and lack of any phenotype [50]. Vasa protein is an essential component of germplasm and represents a poorly understood complex of RNA and proteins that is required for germ cell determination. Null mutation leads to sterility in female mice, resulting from severe defects in oogenesis [52]. On the other hand, Tarin et al., 2000 [23] concluded that oocyte ageing is associated with a distorted secondary sex ratio in favour of males. Our preliminary results with zebrafish (vasa GFP transgenic strain), indicated that oocyte ageing significantly affects the number and development of the primordial germ cells (PGCs). Since depletion of PGCs converts the sex differentiation in favour of males in zebrafish [53] and other fish species [54], the oocyte ageing may bias sex ratio in favour of males or increase the probability of the occurrence of completely sterile individuals. The latter idea can be addressed in future studies.

mRNA levels of transcripts involved in the cell cycle was also investigated upon post-ovulatory and post-stripping oocyte ageing. *CyclinA*, *cyclinB, jnkA* and *jnkB* mRNAs displayed higher abundance in more aged oocytes *in vivo*, while lower levels in more aged oocytes *in vitro*. A similar increasing trend in the abundance of the *cyclinA1*, *cyclinA2* and *JNK1* has been reported in rainbow trout oocytes aged *in vivo* [19]. Similarly decreased mRNA levels of two critical cell cycle-related genes, maturation promoting factor (*MPF*) and mitogen-activated protein kinases (*MAPKs*), have been reported in oocytes aged *in vitro* in porcine [30, 55] and murine models [56]. The latter study indicated the role of critical cell cycle factors and cytoplasmic changes in spontaneous activation of the oocyte ageing. The observed opposite trend towards the increased and decreased mRNA levels of the aforementioned genes during ova ageing *in vivo* and *in vitro* is of interest for future studies.

Apoptotic cell death is the end point of the oocyte ageing process and occurs through caspase activation [57], increased levels of apoptotic signalling protein *Bax*, decreased levels of anti-apoptotic protein *Bcl-xL* [58] and DNA damage [59]. Similarly, in the current study, the genes involved in apoptosis, such as *caspase3A*, *caspase9* and *bax,* exhibited lower levels at the time of ovulation than those in over-ripened eggs at 10-12 HPO and 10 HPS. By contrast, it has been shown that the level of *bax* remains unchanged in the mouse oocytes aged *in vitro*. [60, 61]. At the end of the oocyte ageing time, pro-apoptotic molecules, such as *bax*, induce the release of *cytochrome c*, which activates caspases, while anti-apoptotic molecules, such as *bcl2*, prevent this release [62]. As the expression of the anti-apoptotic protein Bcl2 is decreased during oocyte ageing in mice [61] and pigs [55], the oocytes and developing embryos are more prone to undergo apoptosis [25, 60]. In the present study, the relative mRNA abundance of *bcl2* showed no significant changes during both *in vivo* and *in vitro* oocyte ageing. Bcl2 is known as a family of proteins regulating cell death by either inducing or inhibiting apoptosis [63, 64]. The observed trend for the relative mRNA abundance of *bcl2* in our study might be attributed to the inducing role of the examined gene for apoptosis during ova ageing, while many other genes encoding the Bcl2 protein might have an inhibiting role in the occurrence of apoptosis. Levels of *hsp70* and *cox1* mRNAs showed the increasing tendency at the beginning phase of oocyte ageing, while increased levels of *caspase3A*, *caspase9* and *bax,* were obvious at 10-12 HPO and HPS. Consistent with these observations, Lord et al., 2013 [25] suggested that oxidative stress in aged oocytes can be considered as an early marker of oocyte ageing before the activation of caspase-3 and before the appearance of the morphological features of oocyte ageing and apoptosis. Although they do not differ statistically, levels of *CathepsinB* and *cathepsinZ* mRNAs were both increased during the *in vivo* and *in vitro* egg storage, except for the initial decrease of *cathepsinZ* once the eggs

stored *in vitro*. Upregulation of *cathepsinB* is associated with cell death [65]. Aegerter et al., 2005 [19] also found that *in vivo* oocyte ageing in rainbow trout is associated with the increased mRNA abundance of *cathepsinZ*. Lysosomal proteases *cathepsinD* and *cathepsinB* act as pro-apoptotic mediators of apoptosis [66]. Therefore, the increased mRNA levels of the *cathepsinB* and *cathepsinZ* genes during the oocyte ageing progress might lead to the increased mRNA levels of the apoptotic genes observed in this study.

Our results indicated that the relative mRNA level of *igf2* showed a decreasing trend to 6 HPO and HPS and then increased until the complete loss of egg viability occurred. Aegerter et al., 2005 [19] also found higher quantities of *igf2* mRNAs in more aged rainbow trout oocytes at 14 HPO than that in the freshly ovulated ones. The IGF axis has been shown to play a role in the inhibition of apoptotic cell death [67]. The increased tendency towards the mRNA levels of the *igf2* observed in this study therefore, might be considered as a defence mechanism against the occurrence of over-ripening of the eggs and the apoptosis.

Although examining the mRNA abundance of single target genes could be a good and helpful tool, it is not yet enough to conclude on the possible involvement of oxidative stress in the progress of fish oocyte ageing. In fact, additional analysis such as microarray analysis, total ROS measurement, mitochondrial dysfunction indicators, ATP content of the eggs, etc., are required to fully evaluate the contribution of oxidative stress to the drop of egg quality during postovulatory ageing. Additionally, as there is no clear link between mRNA abundance and protein synthesis in metaphase 2 oocytes, studying the proteome profile changes during the fish oocyte ageing could provide valuable information about the oocyte ageing and its underlying mechanisms. Further analysis of these genes during development in eggs at varying ageing times will be useful and will benefit to the study on fish egg quality.

The epigenetic changes in mouse oocytes have been associated with post-ovulatory ageing [68, 69]. The ageing of oocytes has been shown to significantly alter the methylation pattern of imprinted genes in both mouse oocyte and the developing placenta [69, 70]. This process in turn alters the demethylation events after fertilization [71]. The epigenetic modification related factors (DNA methylation and histone modification) might be involved in defects arising in aged oocytes and the originating embryos. Investigating the epigenetic changes associated with fish oocyte ageing seems to be interesting for the future studies.

CONCLUSIONS

The results obtained in our study demonstrate that oxidative stress is not the main initiator of the oocyte ageing in common carp. However, complementary tests and analysis are required to clearly clarify its involvement. The apoptosis-related genes however, were significantly altered following the prolonged time interval between ovulation and fertilization demonstrating that apoptotic pathway might be involved in the progress of oocyte ageing.

ABBREVIATIONS

CAT: catalase
GPX: glutathione peroxidase
GR: glutathione reductase
HPO: Hours Post Ovulation
HPS: Hours Post Stripping
SOD: superoxide dismutase
TBARS: thiobarbituric acid reactive substances

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REFERENCES

Schultz GA, Heyner S. Gene expression in pre-implantation mammalian embryos. Mutat Res. 1992; 296:17-31.

Minami N, Suzuki T, Tsukamoto S. Zygotic gene activation and maternal factors in mammals. J Reprod Dev. 2007; 53:707-715.

Torres-Padilla ME, Bannister AJ, Hurd PJ, Kouzarides T, Zernicka-Goetz M. Dynamic distribution of the replacement histone variant H3.3 in the mouse oocyte and preimplantation embryos. Int J Dev Biol. 2006; 50:455-461.

Yoshida N, Brahmajisyula M, Shoji S, Amanai M, Perry AC. Epigenetic discrimination by mouse metaphase II oocytes mediated asymmetric chromatin remodelling independently of meiotic exit. Dev Biol. 2007; 301:464-477.

Lam TJ, Nagahama Y, Chan K, Hoar WS. Overripe eggs and post-ovulatory corpora lutea in the three spined stickleback. Can J Zool. 1978; 56:2029–2036.

McEvoy (Barton) L.-A. Ovulatory rhythms and over-ripening of eggs in cultivated turbot, *Scophthalmus maximus* L. J Fish Biol. 1984; 24:437–448.

Rime H, Guitton N, Pineau C, Bonnet E, Bobe J, Jalabert B. Post-ovulatory ageing and egg quality: A proteomic analysis of rainbow trout coelomic fluid. Reprod Biol Endocrinol. 2004; 2:26–36.

Bobe J, Labbe C. Egg and sperm quality in fish. Gen Comp Endocr. 2010;165:535–548.

Samarin AM, Policar T, Lahnsteiner F. Fish oocyte ageing and its effect on egg quality. Rev Fish Sci Aquac. 2015b; 23:302–314.

Lahnsteiner F, Urbanyi B, Horvath A, Weismann T. Biomarkers for egg quality determination in cyprinid fish. Aquaculture. 2001; 195:331–352.

Samarin AM, Gela D, Bytyutskyy D, Policar T. Determination of the best post-ovulatory stripping time for the common carp, Cyprinus carpio. J Appl Ichthyol. 2015a; 31(Suppl. 2):51–55.

Aegerter S, Jalabert B. Effects of post ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout *Oncorhynchus mykiss*. Aquaculture. 2004; 231:59–71.

Bonnet E, Fostier A, Bobe J. Characterization of rainbow trout egg quality: A case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations. Theriogenology. 2007a; 67:786–794.

Samarin AM, Żarski D, Palińska-Żarska K, Krejszeff S, Blecha M, Kucharczyk D, Policar T. In vitro storage of unfertilized eggs of the Eurasian perch and its effect on egg viability rates and the occurrence of larval malformations. Animal. 2017; 11:1,78–83.

Flajshans M, Kohlmann K, Rab P. Autotriploid tench *Tinca tinca* (L.) larvae obtained by fertilization of eggs previously subjected to postovulatory ageing in vitro and in vivo. J Fish Biol. 2007; 71:868–876.

Samarin AM, Blecha M, Uzhytchak M, Bytyutskyy D, Żarski D, Flajshans M, Policar T. 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. 2016; 450:431–438.

Zuccotti M, Merico V, Cecconi S, Redi CA, Garagna S. What does it take to make a developmentally competent mammalian egg? Hum Reprod Update. 2011; 17:525-540.

Aegerter S, Jalabert B, Bobe J. MessengerRNAstockpile of cyclin B, insulin-like growth factor I, insulin-like growth factor II, insulinlike growth factor receptor Ib, and p53 in the rainbow trout oocyte in relation with developmental competence. Mol Reprod Dev. 2004; 67:127-135.

Aegerter S, Jalabert B, Bobe J. Large scale real-time PCR analysis of mRNA abundance in rainbow trout eggs in relationship with egg quality and post-ovulatory ageing. Mol Reprod Dev. 2005; 72:377–385.

Bonnet E, Fostier A, Bobe J. Microarray-based analysis of fish egg quality after natural or controlled ovulation. BMC Genomics. 2007b; 8:55.

Mommens M, Fernandes JM, Bizuayehu TT, Bolla SL, Johnston IA, Babiak I. Maternal gene expression in Atlantic halibut (*Hippoglossus hippoglossus* L.) and its relation to egg quality. BMC Res Notes. 2010; 3:138.

Ma H, Weber GM, Hostuttler MA, Wei H, Wang L, Yao J. MicroRNA expression profiles from eggs of different qualities associated with post-ovulatory ageing in rainbow trout (*Oncorhynchus mykiss*). BMC Genomics. 2015; 16:201.

Tarin JJ, Perez-Albala S, Cano A. Consequences on offspring of abnormal function in ageing gametes. Hum Reprod Update. 2000; 6:532–549.

Takahashi T, Takahashi E, Igarashi H, Tezuka N, Kurachi H. Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. Mol Reprod Dev. 2003; 66:143–152.

Lord T, Nixon B, Jones KT, Aitken RJ. Melatonin prevents postovulatory oocyte aging in the mouse and extends the window for optimal fertilization in vitro. Biol Reprod. 2013; 88:1–9.

Dean RT, Gieseg S, Davies MJ. Reactive species and their accumulation on radical-damaged proteins. Trends Biochem Sci. 1993; 18:437–441.

Headlam HA, Davies MJ. Markers of protein oxidation: different oxidants give rise to variable yields of bound and released carbonyl products. Free Radic Biol Med. 2004; 36:1175–1184.

Orrenius S, Gogvadze V, Zhivotovsky B. Mitochondrial oxidative stress: implications for cell death. Annu Rev Pharmacol Toxicol. 2007; 47:143–183.

Mok HJ, Shin H, Lee JW, Lee GK, Suh CS, Kim KP, Lim HJ. Age-associated lipidome changes in metaphase II mouse oocytes. Plos One. 2016; 11:e0148577.

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

Tarin JJ, Perez-Albala S, Perez-Hoyos P, Cano A. Postovulatory aging of oocytes decreases reproductive fitness and longevity of offspring. Biol Reprod. 2002; 66:495–499.

Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, Dudekula DB, VanBuren V, Ko MSH. Age-associated alteration of gene expression patterns in mouse oocytes. Hum Mol Genet. 2004; 13(19):2263–2278.

Horvath L, Tamas G. Common Carp, part 1: mass production of eggs and early fry. Food and Agriculture Organization of the United Nations. 1985; Rome, 44 pp.

Komrakova M, Holtz W. Factors responsible for successful chilled storage of unfertilized rainbow trout (*Oncorhynchus mykiss*) eggs. Aquaculture. 2009; 286:156-163.

Babiak I, Dabrowski K. Refrigeration of rainbow trout gametes and embryos. J Exp Zool A Comp Exp Biol. 2003; 300(2):140-151.

Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007; 23(10):1289–1291.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3 - new capabilities and interfaces. Nucleic Acids Res. 2012; 40(15):e115.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985; 150:76-85.

Claiborne A. Catalase activity. in: Greenwall, R.A. (Ed.), CRC handbook of methods in oxygen radical research. 1985; CRC Press, Boca Raton, FL: 283-284.

Nishikimi M, Rao NA, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Bioph Res Co. 1972; 46:849-854.

Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller DJ. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney - possible implications in analgesic nephropathy. Biochem Pharmacol. 1984; 33:1801-1807.

Cribb AE, Leeder JS, Spielberg SP. Use of a microplate reader in an assay of glutathione-reductase using 5,5'-dithiobis(2-nitrobenzoic acid). Anal Biochem. 1989; 183:195-196.

Li P, Li ZH, Dzyuba B, Hulak M, Rodina M, Linhart O. Evaluating the impacts of osmotic and oxidative stress on common carp (*Cyprinus carpio* L.) sperm caused by cryopreservation techniques. Biol Reprod. 2010; 83:852–858.

Oliver C, Ahn B, Moerman E, Goldstein S, Stadtman E. Age-related changes in oxidized proteins. J Biol Chem. 1987; 262:5488-5491.

Lord T, Aitken RJ. Oxidative stress and ageing of the post-ovulatory oocyte. Reproduction. 2013; 146:217–227.

Esponda P, Diaz H. Age-induced apoptosis and activation of heat shock protein HSP70 in mammalian oocytes. Signaling and Apoptosis. 2006; PP-184a

Boerjan ML, de Boer P. First cell cycle of zygotes of the mouse derived from oocytes aged postovulation in vivo and fertilized in vivo. Mol Reprod Dev. 1990; 25:155–163.

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum Reprod. 1998;13:998–1002.

Samarin AM, Sampels S, Policar T, Rodina M, Hematyar N, Samarin AM. mRNA abundance changes during in vitro oocyte ageing in African catfish *Clarias gariepinus* (Burchell, 1822). Aquac Res. 2018; 49:1037-1045.

Raz E. 2000. The function and regulation of vasa-like genes in germ-cell development. Genome Biol. 2000; 1(3)

Nikolic A, Volarevic V, Armstrong L, Lako M, Stojkovic M. Primordial Germ Cells: Current Knowledge and Perspectives. Stem Cells Int. 2016; 1741072.

Saffman E, Lasko P. Germline development in vertebrates and invertebrates. Cell Mol Life Sci. 1999; 55:1141–1163.

Tzung KW, Goto R, Saju JM, Sreenivasan R, Saito T, Arai K, Yamaha E, Hossain MS, Calvert MEK, Orban L. Early Depletion of Primordial Germ Cells in Zebrafish Promotes Testis Formation. Stem Cell Rep. 2015; 4(1):61-73.

Liu W, Li SZ, Li Z, Wang Y, Li XY, Zhong JX, Zhang XJ, Zhang J, Zhou L, Gui JF. Complete depletion of primordial germ cells in an All-female fish leads to Sex-biased gene expression alteration and sterile All-male occurrence. BMC Genomics. 2015; 16:971.

Ma W, Zhang D, Hou Y, Li YH, Sun QY, Sun XF, Wang WH. Reduced expression of MAD2, BCL2, and MAP kinase activity in pig oocytes after in vitro aging are associated with defects in sister chromatid segregation during meiosis II and embryo fragmentation after activation. Biol Reprod. 2005; 72:373–383.

Xu Z, Abbott A, Kopf GS, Schultz RM, Ducibella T. Spontaneous activation of ovulated mouse eggs: time-dependent effects on M-phase exit, cortical granule exocytosis, maternal messenger ribonucleic acid recruitment, and inositol 1,4,5-triphosphate sensitivity. Biol Reprod. 1997; 57:743–750.

Takai Y, Matikainen T, Juriscova A, Kim MR, Trbovich AM, Fujita E, Nakagawa T, Lemmers B, Flavell RA, Hakem R, Momoi T, Yuan J, Tilly JL, Perez GI. Caspase-12 compensates for lack of caspase-2 and caspase-3 in female germ cells. Apoptosis. 2007; 12:791-800.

Perez GI, Juriscova A, Matikainen T, Morityama T, Kim MR, Takai Y, Pru JK, Kolesnick RN, Tilly JL. A central role for ceramide in the age-related acceleration of apoptosis in the female germline. FASEB J. 2005; 19:860-862.

Fujino Y, Ozaki K, Yamamasu S, Ito F, Matsuoka I, Hayashi E, Nakamura H, Ogita S, Sato E, Inoue M. DNA fragmentation of oocytes in aged mice. Hum Reprod. 1996; 11:1480-1483.

Takahashi T, Igarashi H, Amita M, Hara S, Matsuo K, Kurachi H. Molecular mechanism of poor embryo development in postovulatory aged oocytes: Mini review. J Obstet Gynaecol Res. 2013; 39:1431–1439.

Gordo AC, Rodrigues P, Kurokawa M. Intracellular calcium oscillations signal apoptosis rather than activation in in vitro aged mouse eggs. Biol Reprod. 2002; 66:1828– 1837.

Li M, Baumeister P, Roy B, Phan T, Foti D, Luo S, Lee AS. ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. Mol Cell Biol. 2000; 20:5096–5106.

Cleary ML, Smith SD, Sklar J. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell. 1986; 47 (1):19–28.

Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science. 1984; 226 (4678): 1097–9.

Houseweart MK, Pennacchio LA, Vilaythong A, Peters C, Noebels JL, Myers RM. Cathepsin B but not cathepsins L or S contributes to the pathogenesis of Unverricht-Lundborg progressive myoclonus epilepsy (EPM1). J Neurobiol. 2003; 56(4):315–27.

Kågedal K, Johansson U, Ollinger K. The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. FASEB J. 2001; 15:1592-1594.

Butt AJ, Firth SM, Baxter RC. The IGF axis and programmed cell death. Immunol Cell Biol. 1999; 77:256–262.

Huang JC, Yan LY, Lei ZL, Miao YL, Shi LH, Yang JW, Wang Q, Ouyang YC, Sun QY, Chen DY. Changes in histone acetylation during postovulatory aging of mouse oocyte. Biol Reprod. 2007; 77:666–670.

Liang XW, Zhu JQ, Miao YL, Liu JH, Wei L, Lu SS, Hou Y, Schatten H, Lu KH, Sun QY. Loss of methylation imprint of Snrpn in postovulatory aging mouse oocyte. Biochem Biophys Res Commun. 2008; 371(1):16–21.

Liang XW, Ge ZI, Wei L, Guo L, Han ZM, Schatten H, Sun QY. The effects of postovulatory aging of mouse oocytes on methylation and expression of imprinted genes at mid-term gestation. Mol Hum Reprod. 2011; 17:562–567.

Mayer W, Nivelelai A, Walter J, Fundele R, Haaf T. Demethylation of the zygotic paternal genome. Nature. 2000; 403:501–502.

CHAPTER 5

THE ROLE OF OXIDATIVE STRESS ON THE PROGRESS OF OOCYTE AGEING IN GOLDFISH *CARASSIUS AURATUS*

Samarin, A.M., Samarin, A.M., Østbye, T.K., Ruyter, B., Sampels, S., Burkina, V., Blecha, M., Policar, T. The role of oxidative stress on the progress of oocyte ageing in goldfish *Carassius auratus*. Manuscript

My share on this work was 40%.

THE ROLE OF OXIDATIVE STRESS ON THE PROGRESS OF OOCYTE AGEING IN GOLDFISH, *CARASSIUS AURATUS* (LINNAEUS, 1758)

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ABSTRACT

Decreasing egg quality following prolonged oocyte storage *in vitro*, known as post-stripping oocyte ageing is a major restricting factor for practical aquaculture purposes. To examine the possible role of oxidative stress on the progress of goldfish *Carassius auratus* oocyte ageing, the relative mRNA levels of selected maternal transcripts were quantified in oocytes collected from 6 females and incubated *in vitro* for 18 hours post stripping (HPS) at 20°C. During the 18 hour-post-stripping ageing of the oocytes, relative mRNA levels of candidate transcripts involved in oxidative injury, mitochondrial function and stress response (*hsp70*, *cox1*, *sodMn, calmodulin*) cell cycles (*cyclinA, cyclinA2, cyclinB* and *jnk*), apoptosis (*ctpb*), reproduction and germ line speciation (*vasa*) and developmental competence (*igf2*) were measured by real time quantitative PCR. Additionally embryo survival rates were monitored at each ageing time. Egg quality was guaranteed up to 3 HPS while complete loss of egg viabilities was observed at 18 HPS. None of the elative mRNA abundance of the examined genes were significantly altered ($P < 0.05$) through oocyte ageing in goldfish. In addition, the amount of thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation, did not change over time following stripping. The activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPX) also remained almost constant during oocyte ageing. The lack of response of both the activities of antioxidant enzymes and their oxidation products during oocyte ageing strengthens the conclusion that oxidative stress is unlikely to be a main factor determining the progress of oocyte ageing in goldfish until at least 18 hours after egg stripping, when complete loss of egg fertilizing ability occurs.

Keywords: *Carassius auratus; embryo survival; mRNA abundance; oocyte ageing; oxidative stress*

1. INTRODUCTION

In fish, ovulated oocytes are arrested at the metaphase II stage of meiotic division. These oocytes then await fertilization by the spermatozoon. Delayed fertilization during this time results in the occurrence of deleterious processes in oocyte quality, known as oocyte ageing, which might occur during either *in vivo* or *in vitro* storage. In fish, delayed spawning in nature and delayed egg collection in capture leads to oocyte ageing and subsequently to the over-ripening phenomenon. The oocyte quality can affect the success of fertilization, embryo quality and later life of the offspring. Oocyte ageing has been identified as the most important factor affecting egg quality after ovulation in several fish species. During oocyte ageing, morphological, physiological, biochemical, cellular, and molecular changes occur inside the eggs and ovarian fluid (Samarin et al. 2015). Decrease in egg viability rates (e.g., Lahnsteiner et al. 2001; Samarin et al. 2011), increase in the occurrence of larval malformations (e.g., Aegerter and Jalabert 2004; Bonnet et al. 2007a; Samarin et al. 2017) and ploidy anomalies (e.g., Flajshans et al. 2007; Samarin et al. 2016) are well-documented following prolonged oocyte ageing. The optimal *in vitro* storage time for the oocytes varies from a few minutes to a few days among different fish species and depends on the storage temperature (Samarin et al. 2015; Linhart et al. 2016).

Oocytes are responsible for embryo development providing the embryo with transcripts and proteins until the onset of zygotic transcription. Therefore, the dependence of early stages of embryogenesis on the maternal mRNAs and proteins is not surprising (Zuccotti et al. 2011). The levels and processing of maternally provided mRNAs and proteins could be impacted not only by genetic effects but also by non-genetic effects such as environmental variables such as oocyte ageing (Bonnet et al. 2007b). Among lower vertebrates, the transcriptomics of egg quality has been most extensively studied in fishes (Lyman-Gingerich and Pelegri 2007; Cerda et al. 2008; Bobe and Labbe 2010). However, the impact on the oocyte transcriptome as a result of ageing processes has received far less attention. Little is known about the molecular changes involved in the progress of fish oocyte ageing (Aegerter et al. 2004; Aegerter et al. 2005; Bonnet et al. 2007b; Mommens et al. 2010; Ma et al. 2015).

In other vertebrates, most authors believe that the onset of ageing in oocytes is associated with an increase in reactive oxygen species (ROS) (e.g., Lord et al. 2013; Lord and Aitken 2013; Tarin et al. 2000; Takahashi et al. 2003). The increase in ROS levels causes oxidative stress, which essentially affects lipids, proteins and nucleic acids in the oocyte. The consequences are ROS-induced mitochondrial dysfunction, ROS-induced lipid alterations and ROS-induced DNA fragmentation followed by impaired embryonic development, ATP depletion and apoptosis (Tarin et al. 2000; Samarin et al. 2018). In addition, some studies on other vertebrates have suggested the involvement of a decline in critical cell cycle factors (Kikuchi et al. 2002) and impaired mitochondrial function in this phenomenon (Tarin et al. 2002).

There have always been difficulties involved in the study of oocyte ageing in higher vertebrates, regarding ethical issues as well as the intrinsic nature of their reproduction biology and the difficulty of collecting adequate numbers of the oocytes. Thus, there are advantages to using fish as model animals to analyse oocyte ageing; in contrast to other animals, fish potentially have a larger number of oocytes. In addition, fish display a vast diversity of reproductive modes in oocyte ageing studies. The current study therefore, examined the role of oxidative stress and apoptosis on the progress of oocyte ageing using goldfish, *Carassius auratus*, as the model animal. Possible alterations in the oxidation status of the oocytes during oocyte ageing was also investigated by measuring TBARS as the marker of lipid oxidation. In addition the role of oxidative stress on the progress of oocyte ageing was assessed by evaluation of the oxidation status of the oocytes during post-ovulatory ageing. The activity

of antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPX) during oocyte ageing were examined. The relative mRNA abundance of the transcripts involved in oxidative damage and stress response and mitochondrial function, genes with roles in fertilization, embryo development, transcriptional regulation and cell cycling as well as those related to the apoptosis were examined during oocyte ageing.

Identifying molecular mechanisms involved in the decline of oocyte quality with the progress of oocyte ageing could have important implications for aspects of basic research and practicaly applications for aquaculture purposes to prevent or delay oocyte ageing. The results of this study may provide a better understanding of the mechanisms involved in the oocyte ageing process which might be beneficial to other vertebrates as well.

2. MATERIALS AND METHODS

2.1. Ethics

The experimental procedures were performed according to the ethical rules of the EU-harmonized Animal Welfare Act of the Czech Republic. The unit is licensed (No. 53100/2013-MZE-17214) according to the Czech National Directive (the Law against Animal Cruelty, No. 246/1992).

2.2. Fish

Brood goldfish (20 male and 20 female) were captured from an earthen pond when the average daily temperature of the water reached 17 °C in the late spring. The fish were then transferred to indoor cylindrical holding tanks (450 L capacity) supplied with water from a recirculating aquaculture system; females and males were housed separately. The photoperiod was adjusted to 14 L: 10 D, and the storage temperature was gradually increased to 20 °C and was maintained for the duration of the experiment. After 2 weeks of acclimation, the females were injected with carp pituitary homogenate (CPH) (0.3 $mg.kg^{-1}$ body weight). This was followed 12 hours later by a second injection of CPH (3.5 $mg.kg^{-1}$ body weight). Male brood fish (41.2 ± 10.6 g body weight, mean ± SD) were given a single muscular injection of CPH (2 $mg.kg^{-1}$ body weight) when the females received their second injection. These procedures were performed according to the method of Horvath and Tamas (1985). The females were examined for ovulation every 3 hours, starting 6 hours after the second injection. The occurrence of ovulation was judged by gentle palpation of the abdomen. If eggs could be removed by applying gentle pressure to the abdomen, the fish were considered to have already ovulated. The inspections were performed every 3 hours thereafter. Six females, which ovulated within 3 hours (58 ± 11.7 g body weight, mean ± SD), were selected randomly and used for the experiment. To examine ovulation and to collect gametes, the fish were anaesthetized with a 0.03 ml/L clove oil water bath to minimize stress and to make them easier to handle (Flajshans et al. 2007).

2.3. *In vitro* egg storage in the ovarian fluid

Stripped ova of 6 females were collected separately and stored in six-well sterile cell culture plates (each well diameter: 3.5 cm). The eggs were stored in two layers deep (Komrakova and Holtz 2009). The eggs were kept in the ovarian fluid, and no solution, artificial media or extender was used. All plates were individually covered with lids and kept in the dark at 20 °C

in the laboratory incubator for 18 hours. To provide a humidified atmosphere, a few plates were filled with water and placed into the storage chambers (Babiak and Dabrowski 2003; Komrakova and Holtz 2009). Stored ova were fertilized at 0 (immediately after stripping), 3, 6, 9, 12 and 18 HPS.

2.4. Artificial fertilization

In total, 20 mature males were used for the experiment. To provide a uniform fertilizing ability for all egg batches, 0.5 ml of milt was collected separately from each of three males, pooled prior to fertilization and then used at each fertilization step. Sperm motility was assessed empirically for each male separately before egg insemination by observing the sperm under a microscope at a magnification of 40x using a glass slide without a coverslip (Fauvel et al. 2010). A total volume of 1.5 ml was then mixed gently and used for fertilization. For each fertilization step, a batch of ~130 eggs from each female was gently dispersed into separate small petri dishes and then fertilized by using 0.15 ml of mixed milt and adding 2 ml of the hatchery water with continued shaking for 3 minutes. Five ml of hatchery water was then poured into each petri dish to wash the eggs. This sperm-to-egg ratio was confirmed to be sufficient to fertilize all the eggs in the preliminary tests.

2.5. Incubation and examining embryo survival rates

After fertilization, the eggs were washed 4-5 times with the hatchery water to remove the extra milt. Then, the petri dishes were left for 5 minutes to ensure that the eggs were attached to their surface. Finally, the plates were placed separately into small rectangular-shaped incubators (each 4.5 L capacity) with recirculating water at 20 ± 0.5 °C and a flow rate of 1 $L.min^{-1}$ through each incubator. The embryo survival rate was assessed as the indices of egg viability considering the presence/absence of the embryo in each egg. After 24 hours of fertilization, embryo survival rates with respect to the total number of initially fertilized eggs were calculated using a stereomicroscope (Nikon SMZ745T, Japan).

2.6. Transcriptome changes during oocyte ageing

2.6. 1. RNA isolation and reverse transcription

At all HPSs, 1 g of the unfertilized eggs was placed in the cryotubes, frozen in liquid nitrogen and then stored in a -80 °C freezer until RNA isolation. Samples were collected in three replicates for each fertilization time.

To prepare the total RNA, an equal weight of 20 eggs was taken from each tube and treated with TRIzol reagent (Invitrogen) in a Precellys24 homogenizer (Bertin Instruments), 5500 rpm for 2×20 sec with a 5 sec interval. RNA was isolated using a PureLink RNA Purification Kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. During RNA isolation, genomic DNA was depleted from the samples using a PureLink DNase Kit (Invitrogen) according to the protocol. The RNA concentration and the RNA purity were assessed using a NanoDrop Spectrophotometer (ND-1000, Thermo scientific).A concentration of 1000 ng RNA was used to generate cDNA using TaqMan reverse transcription Reagents (Life Technologies). Random hexamers were used to prime the reaction. Reverse transcription was performed at 25 °C for 10 min, 48 °C for 1 hour and finally 95 °C for 5 min. Control reactions were run without TaqMan reverse transcriptase and were used as negative controls in the real-time PCR study.

2.6.2. qRT-PCR analysis

Nucleotide sequences corresponding to goldfish mRNA (NCBI) were used for primer design (by Primer3) (Koressaar & Remm, 2007; Untergasser et al. 2012). Transcript levels of 16 genes (*hsp70*, *cox1*, *sod, gpx1*, *cyclinA, cyclinB, jnkA*, *jnkB*, *caspase3A*, *caspase9*, *bax*, *bcl2, cathepsinB, cathepsinZ*, *vasa* and *igf2*) were determined by real time qPCR in duplicate using the LightCycler 480 (Roche). The reaction mix for the qPCR consisted of 4 µl diluted (1:10) cDNA, 1 µl forward and reverse primer (final concentration of 500 nM, Table 1), and 5 µl SYBR Green-I Master (Roche Applied Science, Germany). All primers were provided by Invitrogen. A standard curve was included for each primer pair to evaluate the primer efficiency. All samples were analysed in parallel, and a non-template control with water substituted for cDNA was run for each primer pair. The qPCR reaction was run under the following conditions: preincubation at 95 °C for 5 minutes, amplification with 45 cycles at 95 °C for 15 seconds and 60 °C for 1 minutes, melting curve at 95 °C for 5 seconds and 65 °C for 1 minute, and cooling at 40 °C for 10 seconds.

The comparative CT method was used for relative quantification of mRNAs. To normalize relative mRNA levels, three reference genes, Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), 18S ribosomal RNA (*18s*) and beta-actin (*b-actin*), were tested. The mRNA abundance of *b-actin* was shown to be highly stable in goldfish eggs collected at different time points after ovulation, and it did not show any significant change in the Ct values of the eggs at different HPSs. Since *b-actin* was the most stable, it was used as the reference gene. Relative mRNA level was then calculated according to the equation $2^{-\Delta Ct}$. Sequences of primers used are listed in Table 1.

Table 1. *qRT-PCR primer sequences.*

Target gene	*Forward primer (5'-3')*	*Reverse primer (5'-3')*	*Genbank accession no.*
hsp 70	CTGTACGAGGGCATCGACTT	GCTTTCTCCACAGGCTCAAG	DQ872648
cytochrome c oxidase I	CTCATTGGAGGATTCGGAAA	GGAATGATGGGGGAAGAAGT	JF752338
vasa	CAGAGGAGGATCACGAGGAG	TTGGCCTTCATCTTTCCAAC	AY821684
igf 2	TGTGGAAAGGCAAACAATGA	CTAGTTCTCCACCGCAAAGG	FJ410929
sod Mn	TTATGCAGCTTCACCACAGC	ACACATCACCCTTTGCCAGT	JX477243
cyclinA	TGCATGTCTGTCCTGAGAGG	TCCACTTCCGGAGGATACAC	EU380204
jnk 1	GACTCCACGTTCACCGTTTT	CGTTCAAGGACATGGTCGTA	EU374209
b-actin	CCCTGTATGCTTCAGGTCGT	ATTGCATGGGGAAGAGCATA	AB039726
calmodulin	TGAAGTGGATGCTGATGGAA	TCTGATCTCCTCCTCGCTGT	JX477193
Sod CuZn	GTCAGACACGTCGGAGACCT	GTATTCCCCAAACAGGGTCA	JX477242
18s ribosomal RNA	CGAGGCCCTTTAATTGGATT	TTCATACGCAATTGGAGCTG	AF047349
CathepsinB	TTCTGGAGCTCTGACGGTCT	GCCATTCACATGATGCTCAC	JX477223
cyclinB	AAGTTCAGGCTGCTTCAGGA	AAGCTGGAGCTGCTTCTTTG	AF273495
sod	GTCAGACACGTCGGAGACCT	GTATTCCCCAAACAGGGTCA	JQ776518
cyclinA2	TGGTGAGCTGAGCTTGATTG	CTCCTGCAATTGTGTGGTTG	AF273493

2.7. Antioxidant enzymes activity assays

2.7.1. Preparation of post-mitochondrial supernatant

Samples of fish eggs (approx. 400 mg) were homogenized using a Tissue Lyser II (Quagen) in 0.1 M K-phosphate buffer (pH 7.4). Homogenates were centrifuged at 15,000 g at 4 °C for 15 min in a Micro 200 R. Supernatants were removed, kept at 0–4 °C and used for protein determination and enzyme assays. Protein levels were measured spectrophotometrically using bovine serum albumin as a standard (Smith et al., 1985). The samples were diluted to a protein content of 10 mg/mL.

2.7.2. Antioxidant enzyme activity

Catalase (CAT) activity was determined as a decrease in hydrogen peroxide in a 96-well flat-bottomed UV-transparent microtitre plate. CAT activity was assessed spectrophotometrically at 240 nm and following the method of Claiborne (1985). Calculations were made using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

The superoxide dismutase (SOD) activity was determined by the method of Nishikimi et al. (1972). Superoxide dismutase activity was assessed spectrophotometrically at 420 nm and expressed as the nitro blue tetrazolium formation per milligram of protein per minute.

Glutathione peroxidase (GPX) activity was measured using the method of Mohandas et al. (1984). Briefly, incubation mixture (0.3 mL) contained post-mitochondrial supernatant (approx. 0.2 mg of protein), K-phosphate buffer 0.05 M (pH 7.0), in EDTA 1 mM, sodium azide 1 mM and glutathione reductase from baker's yeast (7.5 ml from stock containing 1 U/ml), reduced glutathione 4 mM and NADPH 0.8 mM. The reaction was started via the addition of 0.5 mM of hydrogen peroxide. The oxidation of NADPH was recorded at 340 nm. Calculations were made using the molar extinction coefficient 6220 M^{-1} cm^{-1}.

The glutathione reductase (GR) activity was measured by the method of (Cribb et al. 1989).

Briefly, incubation mixture (0.25 mL) contained post-mitochondrial supernatant (approx. 0.3 mg of protein), K-phosphate buffer 0.05 M (pH 7.0), NADPH (0.4 mM), oxidized glutathione (0.4 mM) and diethylenetriaminepentaacetic acid (DTPA) (1 mM). Disappearance of NADPH was measured at 340 nm and calculated as NADPH oxidized formation using molar extinction coefficient of 6220 M^{-1} cm^{-1}.

All assays were performed spectrophotometrically in triplicate or quadruplicate using a 96-well microplate reader (Tecan infinite M200, Germany). Samples were held on ice; measurements were made at 25 °C. Variation in absorbance at each reaction well was linear over time (R^2>0.8). Enzyme activity was expressed as units (μmol for CAT, SOD and GR; nmol for GPX) of substrate oxidized per mg of protein per minute (mean ± SD).

2.8. Thiobarbituric Acid Reactive Substances

The thiobarbituric acid reactive substances (TBARS) method was used to evaluate oocyte lipid peroxidation according to Li et al. (2010). After reaction with thiobarbituric acid in darkness for 15-20 hours (overnight) at room temperature (20 °C), the reaction complex was detected at a wavelength of 530 nm against the sample blank using a UV-visual plate reader (AF 2200; Austria). The results were expressed as equivalents to malonaldehyde (MDA) in μg/g.

2.9. Statistical analysis

The normality of the data was ascertained using SPSS software version 18. Differences between the means of the groups for the embryo survival rates as well as each individual gene, lipid oxidation levels and antioxidant enzyme activities were evaluated using ANOVA followed by Duncan's multiple range test. $P < 0.05$ was considered to be significant.

3. RESULTS

3.1. Embryo survival rates during *in vitro* ova ageing

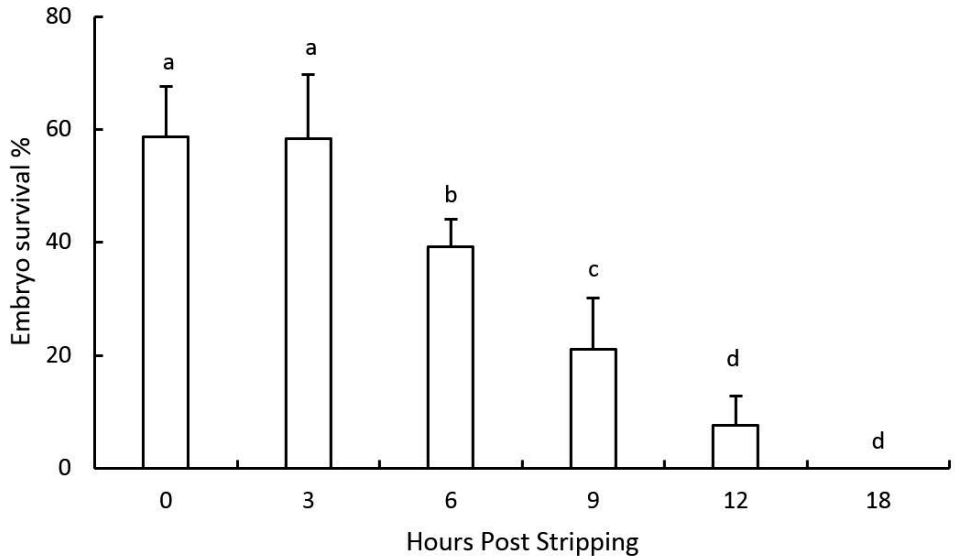

Fig. 1. *Effect of in vitro oocyte ageing on the embryo survival rates in goldfish (mean ± SD). Means sharing a common alphabetical symbol do not differ significantly.*

The embryo survival rates remained nearly constant, at approximately 60%, for the eggs fertilized up to 3 hours post-stripping (Fig. 1). Then, the values decreased significantly over time and were 7.5% for the eggs stored *in vitro* for 12 HPS. After 18 hours of oocyte storage, complete loss of egg fertilizing ability occurred, and no viable embryos were detected.

3.2. Relative abundance of mRNAs during *in vitro* ova ageing

The mRNA levels of 13 selected transcripts were quantified and compared in freshly ovulated and at different hours of post-stripping in aged oocytes, usibg *b-actin* as the reference gene. During 18 hours of post-stripping ova ageing, none of the examined genes showed any significant changes in their mRNA abundance, although most of them showed a trend towards upregulation (Fig. 2). Levels of *cyclinA2*, *cyclinB* and *jnk1* showed continuous down- and upregulated trends during the time interval between egg stripping and the occurrence of oocyte over-ripening.

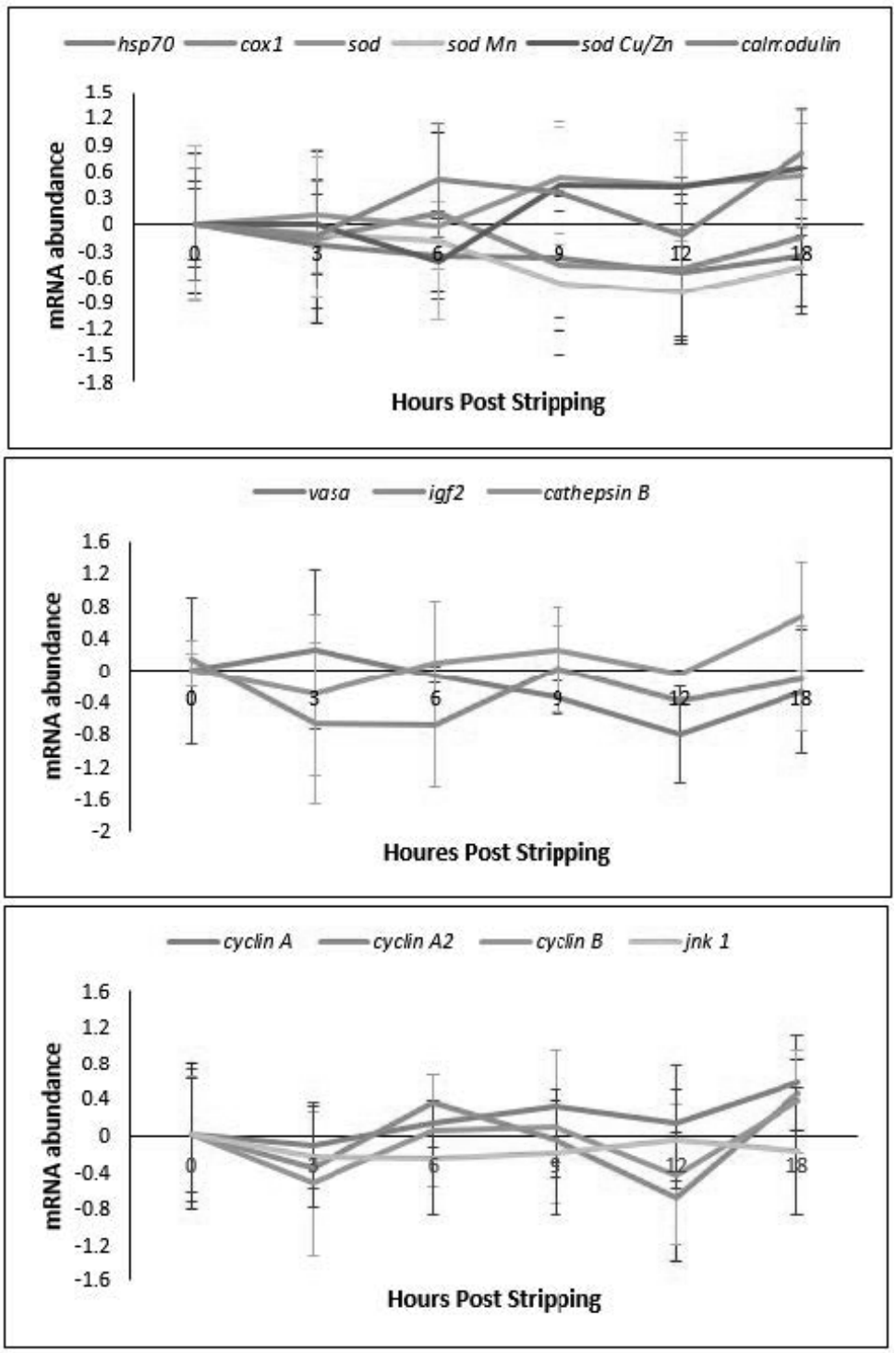

Fig. 2. *Effect of in vitro oocyte ageing at 20 °C on the mRNA expression levels of the selected genes in goldfish (mean ± SD).*

3.3. Effect of in vitro oocyte ageing on the activity of antioxidant enzymes

The activities of SOD and GR did not show any significant changes through post-stripping oocyte ageing (Fig. 3). The CAT and GPX activities decreased at 3 HPS and thereafter stayed almost constant with the prolonged *in vitro* storage of the oocytes.

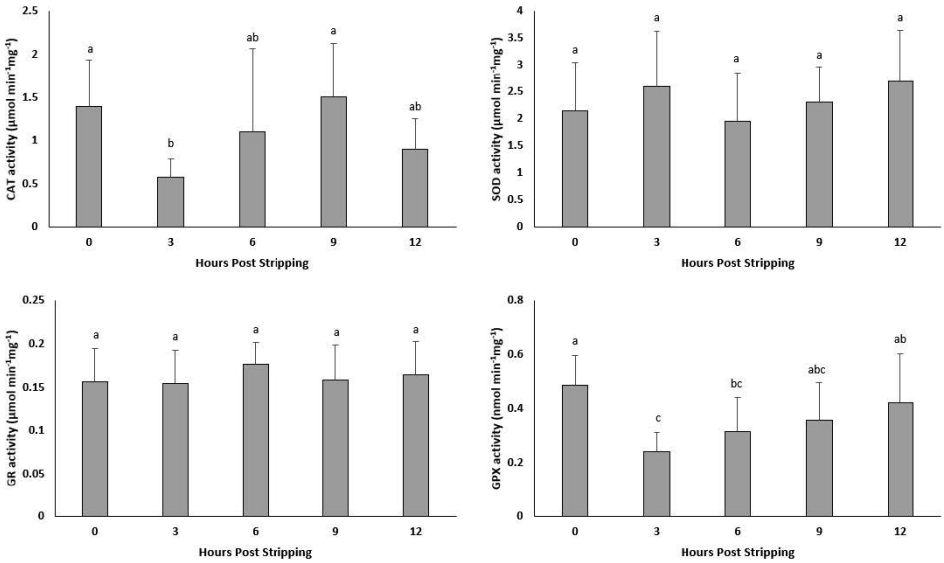

Fig. 3. *Effect of in vitro oocyte ageing in goldfish on the activities of catalase (CAT) (µmol/min/mg), superoxide dismutase (SOD) (µmol/min/mg), glutathione reductase (GR) (µmol/min/mg) and glutathione peroxidase (GPX) (µmol/min/mg) (mean ± SD).*

3.4. Effect of in vitro oocyte ageing on the lipid oxidation of the oocytes

The level of TBARS did not show any significant changes during post-stripping oocyte ageing (Fig. 4).

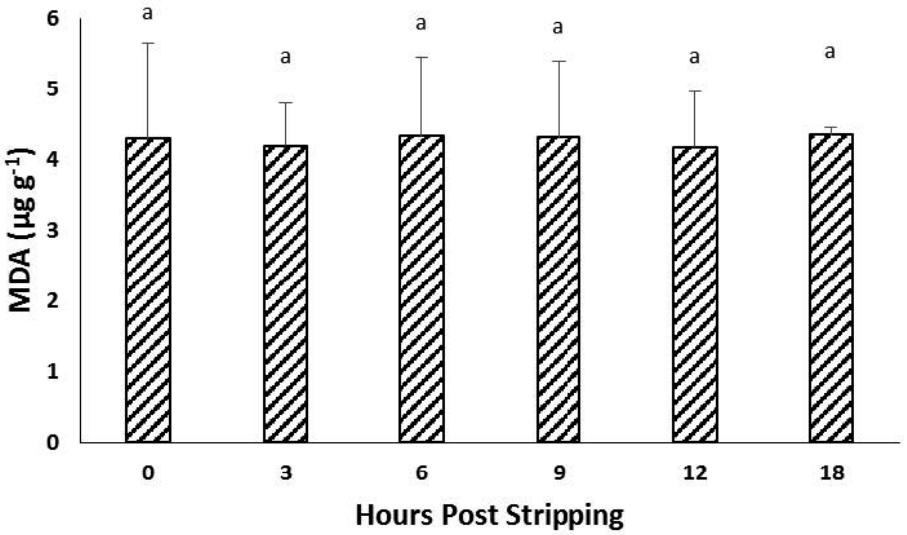

Fig. 4. *Effect of in vitro oocyte ageing in goldfish on TBARS, expressed as malonaldehyde (MDA) (µg g^{-1}) (mean ± SD).*

4. DISCUSSION

Oocyte ageing in goldfish was associated with decreasing embryo survival rates. While the highest survival rates were obtained for the eggs fertilized up to 3 HPS, the complete loss of egg fertilizing ability occurred at 18 HPS. This is in accordance with the results obtained by Formacion et al. (1995), who found that the over-ripening of goldfish eggs occurs at approximately 12 hours post ovulation with advanced degeneration by 24 hours. The successful egg storage time differs from a few minutes to a few weeks depending on the fish species and the storage temperature (Samarin et al. 2015). For instance, the time period after stripping during which egg fertilizing ability is guaranteed has been reported as 1 hour at 18 °C in curimata (*Prochilodus marggravii*) (Rizzo et al. 2003), 2-4 hours at 15 °C in Siberian sturgeon (*Acipense baeri*) and sterlet (*Acipenser ruthenus*) (Gisbert and Williot, 2002), and 3–9 days at 2–12 °C in rainbow trout (*Oncorhynchus mykiss*) (Bonnet et al. 2003; Babiak and Dabrowski; 2003, Niksirat et al., 2007)

It is not clear whether the oocyte ageing process starts by an initiating factor and then follows with a series of events that finally lead to loss of the fertilizing ability or whether different cellular and molecular events start separately and then join each other to complete the process of over-ripening. Most authors believe that the onset of ageing in oocytes is associated with an increase in ROS and consequently increasing oxidative stress (e.g., Lord et al. 2013; Lord and Aitken 2013; Takahashi et al. 2003; Tarin et al. 2000). In addition, many genes used to predict egg quality of aged oocytes in other vertebrates are associated with mitochondrial function, metabolism and cell cycle control (Grondahl et al. 2010; Steuerwald et al. 2007; Pan et al. 2005; Hamatani et al. 2004). Microarray analysis of egg transcriptomic profiles during oocyte ageing have revealed differentially expressed genes involved in mitochondrial function and oxidative stress in mice (Hamatani et al. 2004). Additionally, this study has suggested the alteration of expression patterns for the genes involved in chromatin structure, DNA methylation, genome stability and RNA helicases during oocyte ageing. Comparatively, only a few studies have analysed transcriptomes of egg quality associated with oocyte ageing in fish (Aegerter et al. 2005; Bonnet et al. 2007b; Ma et al. 2015). In

rainbow trout eggs, ova ageing results in the downregulated expression of specific microRNAs and their target genes. These genes are mainly involved in cell death and signal transduction, stress response and DNA damage, RNA degradation, and energy and transcription regulation (Ma et al. 2015).

Our results showed no significant changes in the mRNA levels of oxidative stress related genes (*hsp70*, *cox* and *sod*) during the progress of oocyte ageing in goldfish. Very often, genes involved in oxidative stress and mitochondrial function show an upward trend in expression during oocyte ageing. Esponda and Diaz (2006) reported increased *hsp70* mRNA levels in the aged oocytes. Verbeke et al. (2001) also found that the accumulation of damaged, oxidized and glycerated proteins might result from age-associated defects in the production of heat shock proteins. Similarly, upregulation of *cox1* in mouse oocytes undergoing maternal ageing has been reported (Hamatani et al. 2004). A mitochondrial gene, *cox*, is involved in stress responses that is known to catalyse the electron transfer from reduced cytochrome c to oxygen in the respiratory chain. Disrupted expression of *cox* in aged oocytes may cause an abnormal mitochondrial respiratory chain and thereby affect egg quality. *Sod* is an important antioxidant defence in cells (Sturtz et al. 2001; Pechenino and Brown, 2006) and plays a key role in maintaining cellular homeostasis by removing ROS. *Sod*, an oxidative stress related gene, is known to provide protection against oxidative damage. Decreased mRNA levels of *sod* may decrease the capacity to cope with oxidative damage in oocytes with increasing time following ovulation. However, as we did not find such trends, we suggest that oxidative injury is not a major factor in oocyte overripening in goldfish.

Additionally, the amount of TBARs (the main indicator of lipid peroxidation) did not change in the oocytes over time following stripping. Our results also indicated no significant changes in the activity of CAT, SOD and GPX during post-ovulatory ageing of goldfish oocytes, confirming that oxidative stress is not likely the main initiator in the progress of oocyte ageing in goldfish. The enzymatic antioxidant system can scavenge ROS and therefore, decreases the effect of oxidative stress. If oxidative stress was the initiator of deleterious effects during post-ovulatory oocyte ageing, then an alteration of antioxidant enzymes activity and oxidation markers should occur following ovulation. In African catfish *Clarias gariepinus*, similar results were obtained (Samarin et al. 2018), showing no involvement of oxidative stress on the oocyte ageing process at least until the complete loss of egg fertilizing ability.

In the present study, the mRNA levels of *calmodulin* showed an upward trend during oocyte ageing which was , however, not significant. ROS can directly affect the Ca^{2+} handling system (Wesson and Elliot 1995). Additionally, oxidation affects calcium binding in calmodulin and leads to perturbed calcium homeostasis in aged oocytes (Schallreute et al. 2007). It has been shown that there is impairment of the Ca^{2+} reuptake by Ca^{2+} ATPases in the endoplasmic reticulum (ER) of the aged mouse oocyte (Igarashi et al. 1997). Ca^{2+} release from the 1,4,5-triphosphate (InsP3)-sensitive Ca^{2+} stores decreases in aged mouse oocytes due to depletion of the ER Ca^{2+} store (Takahashi et al. 2000). In other vertebrates, calcium oscillation changes during fertilization showing higher frequency but lower amplitude in more aged oocytes (e.g., Takahashi et al. 2009; Takahashi et al. 2003). The molecular mechanisms of this phenomenon are probably associated with impaired Ca^{2+} regulation (Takahashi et al. 2003). The increased frequency of the calcium oscillations during fertilization in aged oocytes might be related to the higher amount of calcium which has been reported in more aged oocytes (Bahrekazemi et al. 2010; Craik and Harvey, 1984). Takahashi et al. (2003) indicated that the altered Ca^{2+} oscillation led to poor embryo development through affecting the information required for oocyte development.

Although they do not differ significantly, the relative mRNA levels of the transcripts involved in cell cycling (*cyclinA*, *cyclinA2*, *cyclinB* and *jnk1*) were continuously decreased and increasde

during the time interval between egg collection until the occurrence of oocyte ageing. An increasing trend in the relative mRNA abundance of *cyclinA1*, *cyclinA2* and *JNK1* has been reported in rainbow trout oocytes aged *in vivo* (Aegerter et al. 2005). On the other hand, decreased mRNA levels of two critical cell cycle-related genes, maturation promoting factor (*MPF*) and mitogen-activated protein kinases (*MAPKs*), have been reported in oocytes aged *in vitro* in porcine (Ma et al. 2005; Kikuchi et al. 2002) and mouse models (Xu et al. 1997). The latter study indicated the role of critical cell cycle factors and cytoplasmic changes in the spontaneous activation of oocyte ageing. The observed trend towards the continuous increase and decrease mRNA levels of the abovementioned transcripts during ova ageing is interesting for future studies.

The downward trend observed in the mRNA levels of *vasa* in our study during *in vitro* oocyte ageing is consistent with a previous study indicating the expression of *vasa* mRNA is downregulated with maternal ageing in mouse oocytes (Hamatani et al. 2004). *Vasa* is a gene involved in the development of primordial germ cells (PGCs), and its activity is required for the differentiation of the germ cells into gametes, (Raz, 2000) as well as the functionality of germ cells (Nikolic et al., 2016). Loss of *vasa* function in the mouse affects the differentiation of the male germ cells resulting in male sterility and the lack of any phenotype (Raz, 2000). Vasa protein is an essential component of germplasm and represents a poorly understood complex of RNA and proteins that is required for germ cell determination. Null mutations lead to sterility in female mice resulting from severe defects in oogenesis (Saffman and Lasko, 1999). On the other hand, Tarin et al. (2000) concluded that oocyte ageing is associated with a distorted secondary sex ratio in favour of males. Our preliminary results with zebrafish (vasa GFP transgenic strain), indicated that oocyte ageing significantly affects the number and development of the primordial germ cells (PGCs) (unpublished data). Since depletion of PGCs converts the sex differentiation in favour of males in zebrafish (Tzung et al. 2015) and other fish species (liu et al. 2015), oocyte ageing may bias the sex ratio in favour of males or the occurrence of completely sterile individuals. This possibility should be addressed in future studies.

Our results indicate that the relative mRNA abundance of *igf2* decreased slightly up to 6 HPS and then showed an increasing trend until the complete loss of egg viabilities occurred. Aegerter et al. (2005) also found higher quantities of *igf2* mRNAs in more aged rainbow trout oocytes at 14 HPO compared to freshly ovulated ones. The IGF axis has been shown to play a role in the inhibition of apoptotic cell death. Therefore, the increased mRNA levels of *igf2* observed in this study might be considered a defence mechanism against the occurrence of over-ripening of the eggs and apoptosis. Apoptotic cell death is the end point of the oocyte ageing process. With elapsing time after stripping, the mRNA expression of *cathepsinB* also increased. Upregulation of *cathepsinB* has been shown to be associated with cell death (Houseweart et al. 2003). Aegerter et al. (2005) also found that *in vivo* oocyte ageing in rainbow trout was associated with the increased mRNA abundance of *cathepsinZ*. In African catfish *Clarias gariepinus* the *in vitro* oocyte ageing resulted in an upward trend in the mRNA level of *cathepsinD*, an apoptosis-related gene (Samarin et al. 2018). Lysosomal proteases *cathepsinD* and *cathepsinB* act as pro-apoptotic mediators of apoptosis (Kågedal et al. 2001). Therefore, the observed increasing trend in the mRNA levels of *CathepsinB* was not unexpected in the current study. Our recent experiment on common carp indicated the role of apoptotic related genes on the progress of oocyte ageing (unpublished data).

Although examining the mRNA abundance of single target genes could be a good and helpful tool, it is not yet enough to conclude on the possible involvement of oxidative stress in the progress of fish oocyte ageing. In fact, additional analysis such as microarray analysis, total ROS measurement, mitochondrial dysfunction indicators, ATP content of the eggs, etc.,

are required to fully evaluate the contribution of oxidative stress to the drop of egg quality during postovulatory ageing. Additionally, as there is no clear link between mRNA abundance and protein synthesis in metaphase 2 oocytes, studying the proteome profile changes during the fish oocyte ageing could provide valuable information about the oocyte ageing and its underlying mechanisms. Further analysis of these genes during development in eggs at varying ageing times will be useful and will benefit to the study on fish egg quality.

It is believed that in fish embryo development, the transition from maternal genes to the zygotic genome occurs during the mid-blastula stage (Tadros and Lipshitz 2009). Since transcription of the maternal genome is silent from prophase I of the first meiotic cell division up to zygotic genome activation, cytoplasmic regulation of mRNA adenylation is an important mechanism of post-transcriptional control in oocytes (e.g., Gebauer et al. 1994; Paynton and Bachvarova, 1994; Paynton et al. 1988). Maternal effect genes encode oocyte proteins that play important roles in orchestrating the early stages of embryogenesis. Until zygotic genome activation, the protein levels can be regulated by the Poly-A tail length of maternal genes, since transcription of the maternal genome is repressed at the onset of meiotic progression (e.g., Clarke, 2012; Weill et al. 2012; Kang and Han, 2011). Delayed fertilization in aged oocytes may affect this post-transcriptional regulation followed by developmental defects. Recently, oocyte ageing in murine metaphase II oocytes has been shown to be associated with the shortening of the poly-A tails of maternal effect genes in either *in vivo* or *in vitro* culture media (Dankert et al. 2014). The authors concluded that Poly-A tail shortening can, in turn, affect the protein translation time of maternal gene transcripts followed by disturbed fertilization and developmental defects. Moreover, a study in the Western clawed frog *Xenopus tropicalis* has indicated that the 3-hour post-ovulatory ageing of the oocytes leads to deadenylation of transcripts involved in translation and energy metabolism, and a consequent decrease in fertilization rates and an increase in mortality and malformation (Kosubek et al. 2010). The authors suggested that a decrease in the relative abundance of polyadenylated mRNA could be a result of either deadenylation or degradation of maternal mRNA. They supposed that the poor translation of deadenylated mRNAs in frog aged oocytes is responsible for the reduced synthesis of specific proteins that can contribute to the loss of their developmental potential. Recent studies on other vertebrates have also demonstrated that post-ovulatory oocyte ageing can affect the later life of the offspring through epigenetic changes. Therefore, an altered epigenetic profile, which would be able to change gene expression in embryos arising from aged oocytes, might be responsible for some of the abnormalities observed in the later life of the offspring (Lord & Aitken 2013). No study has yet been performed on the effect of fish oocyte ageing on the subsequent growth and genetic structure of progeny. This topic can be addressed in future studies.

5. CONCLUSIONS

The results of the current study indicate that oxidative stress is not likely the initiator of the oocyte ageing process in goldfish until at least 18 hours after egg stripping, when the complete loss of egg fertilizing ability occurs.

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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, 59-71. http://dx.doi.org/10.1016/j.aquaculture.2003.08.019

Aegerter, S., Jalabert, B., Bobe, J., 2004. MessengerRNAstockpile of cyclin B, insulin-like growth factor I, insulin-like growth factor II, insulinlike growth factor receptor Ib, and p53 in the rainbow trout oocyte in relation with developmental competence. Mol. Reprod. Dev. 67, 127–135. http://dx.doi.org/10.1002/mrd.10384

Aegerter, S., Jalabert, B., Bobe, J., 2005. Large scale real-time PCR analysis of mRNA abundance in rainbow trout eggs in relationship with egg quality and postovulatory ageing. Mol. Reprod. Dev. 72, 377–385. http://dx.doi.org/10.1002/mrd.20361.

Babiak, I., Dabrowski, K., 2003. Refrigeration of rainbow trout gametes and embryos. J. Exp. Zool. A Comp. Exp. Biol. 300, 140–151. http://dx.doi.org/10.1002/jez.a.10319

Bahrekazemi, M., Soltani, M., Matinfar, A., Abtahi, B., Pusti, I., Mohagheghi Samarin, A., Mojazi Amiri, B., 2010. Biochemical and histological studies of over-ripened oocyte in the Caspian brown trout (Salmo trutta caspius) to determine biomarkers for egg quality. Iran. J. Fish. Sci. 9 (1), 33–48.

Bobe, J., Labbe, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165, 535-548. http://dx.doi.org/10.1016/j.ygcen.2009.02.011.

Bonnet, E., Jalabert, B., Bobe, J., 2003. A 3-day *in vitro* storage of rainbow trou *Oncorhynchus mykiss* unfertilised eggs in coelomic fluid at 12 °C does not affect development success. Cybium 27, 47–51.

Bonnet, E., Fostier, A., Bobe, J., 2007a. Characterization of rainbow trout egg quality: a case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations. Theriogenology 67, 786–794. http://dx.doi.org/10.1016/j.

Bonnet, E., Fostier, A., Bobe, J., 2007b. Microarray-based analysis of fish egg quality after natural or controlled ovulation. BMC Genomics. 8, 55. https://doi.org/10.1186/1471-2164-8-55

Cerda, J., Bobe, J., Babin, P.J., Admon, A., Lubzens, E., 2008. Functional genomics and proteomic approaches for the study of gamete formation and viability in farmed finfish. Rev. Fish. Science. 16, 54–70. https://doi.org/10.1080/10641260802324685

Claiborne, A., 1985. Catalase activity. In R. A. Greenwall (Ed.), CRC handbook of methods in oxygen radical research (pp. 283–284). Boca Raton, FL: CRC Press.

Clarke, H.J., 2012. Post-transcriptional control of gene expression during mouse oogenesis. Results and Problems in Cell Differentiation 55: 1–21.

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, 115-134. http://dx.doi.org/10.3923/jfas.2009.306.315

Cribb, A.E., Leeder, J.S., Spielberg, S.P., 1989. Use of a microplate reader in an assay of glutathione-reductase using 5,5'-dithiobis (2-nitrobenzoic acid). Anal. Biochem. 183, 195-196. PMID: 2619044

Dankert, D., Demond, H., Trapphoff, T., Heiligentag, M., Rademacher, K., Eichenlaub-Ritter, U., Horsthemke, B., Grümmer, R., 2014. Pre- and Postovulatory Aging of Murine Oocytes Affect the Transcript Level and Poly(A) Tail Length of Maternal Effect Genes. PLoS One. 1;9(10):e108907. http://dx.doi.org/10.1371/journal.pone.0108907.

Esponda, P., Dıaz, H., 2006. Age-induced apoptosis and activation of heat shock protein HSP70 in mammalian oocytes. Signaling and Apoptosis. PP-184a.

Fauvel, C., Suquet, M., Cosson, J., 2010. Evaluation of fish sperm quality. J. Appl. Ichthyol. 26 (5), 636-643. http://dx.doi.org/10.1111/j.1439-0426.2010.01529.x.

Flajshans, M., Kohlmann, K., Rab, P., 2007. Autotriploid tench *Tinca tinca* (L.) larvae obtained by fertilization of eggs previously subjected to postovulatory ageing in vitro and *in vivo*. J. Fish Biol. 71, 868–876. http://dx.doi.org/10.1111/j.1095-8649.2007.01557.x.

Formacion, M.J., Venkatesh, B., Tan, C.H., Lam, T.J., 1995. Overripening of ovulated eggs in goldfish, *Carassius auratus*: II. Possible involvement of postovulatory follicles and steroids. Fish Physiol. Biochem. 14 (3), 237-246. http://dx.doi.org/10.1007/BF00004314.

Gebauer, F., Xu, W., Cooper, G.M., Richter, J.D., 1994. Translational control by cytoplasmic polyadenylation of c-mos mRNA is necessary for oocyte maturation in the mouse. EMBO J 13, 5712-5720. PMC395537

Gisbert, E., Williot, P., 2002. Influence of storage duration of ovulated eggs prior to fertilization on the early ontogenesis of Sterlet, *Acipenser ruthenus*, and Siberian sturgeon, A. Baeri. Int. Rev. Hydrobiol. 87, 605-612. http://dx.doi.org/10.1002/1522-2632(200211)

Grondahl, M.L., Yding Andersen, C., Bogstad, J., Nielsen, F.C., Meinertz, H., Borup, R., 2010. Gene expression profiles of single human mature oocytes in relation to age. Hum. Reprod. 25(4), 957-68. http://dx.doi.org/10.1093/humrep/deq014

Hamatani, T., Falco, G., Carter, M.G., Akutsu, H., Stagg, C.A., Sharov, A.A., Dudekula, D.B., VanBuren, V., Ko, M.S.H., 2004. Age-associated alteration of gene expression patterns in mouse oocytes. Human Molecular Genetics. 13(19) 2263-2278. https://doi.org/10.1093/hmg/ddh241

Horvath, L., Tamas, G., 1985. Common Carp, part 1: mass production of eggs and early fry. Food and Agriculture Organization of the United Nations, Rome, 44 pp.

Houseweart, M.K., Pennacchio, L.A., Vilaythong, A., Peters, C., Noebels, J.L., Myers, R.M., 2003. Cathepsin B but not cathepsins L or S contributes to the pathogenesis of Unverricht-Lundborg progressive myoclonus epilepsy (EPM1). J Neurobiol. 56 (4), 315-327. http://dx.doi.org/10.1002/neu.10253

Igarashi, H., Takahashi, E., Hiroi, M., Doi, K., 1997. Aging-related changes in calcium oscillations in fertilized mouse oocytes. Mol. Reprod. Dev. 48, 383-390. https://doi.org/10. 1002/(ISSN)1098-2795

Kågedal, K., Johansson, U., Öllinger, K., 2001. The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. FASEB J. 15, 1592-1594. https://doi.org/10.1096/fj.00-0708fje

Kang, M.K., Han, S.J., 2011. Post-transcriptional and post-translational regulation during mouse oocyte maturation. BMB. Rep. 44, 147-157. http://dx.doi.org/10.5483/BMBRep.2011.44.3.147

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, 211-222. https://doi.org/10.1089/15362300260339494

Komrakova, M., Holtz, W., 2009. Factors responsible for successful chilled storage of unfertilized rainbow trout (*Oncorhynchus mykiss*) eggs. Aquaculture 286, 156-163. https://doi.org/10.1016/j.aquaculture.2008.09.019

Koressaar, T., Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics. 23(10), 1289-1291. https://doi.org/10.1093/bioinformatics/btm091

Kosubek, A., Klein-Hitpass, L., Rademacher, K., Horsthemke, B., Ryffel, G.U., 2010. Aging of Xenopus tropicalis eggs leads to deadenylation of a specific set of maternal mRNAs and loss of developmental potential. PLoS ONE. 5(10), e13532. https://doi.org/10.1371/journal. pone.0013532

Lahnsteiner, F., Urbanyi, B., Horvath, A., Weismann, T., 2001. Bio-markers for egg quality determination in cyprinid fish. Aquaculture 195, 331–352. http://dx.doi.org/10. 1016/S0044-8486(00)00550-0.

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. Biol. Reprod. 83, 852-858. https://doi.org/10. 1095/biolreprod.110.085852

Linhart, O., Shelton, W.L., Tuckova, V., Rodina, M., Siddique, M.A.M., 2016. Effects of Temperature on In Vitro Short-Term Storage of Sterlet Sturgeon (*Acipenser Ruthenus*) Ova. Reprod. Domest. Anim. 51, 165-170. http://dx.doi.org/10.1111/rda.12661

Liu, W., Li, S.Z., Li, Z., Wang, Y., Li, X.Y., Zhong, J.X., Zhang, X.J., Zhang, J., Zhou, L., Gui, J.F., 2015. Complete depletion of primordial germ cells in an All-female fish leads to Sex-biased gene expression alteration and sterile All-male occurrence. BMC Genomics. 16, 971. http://dx.doi.org/10.1186/s12864-015-2130-z

Lord, T., Aitken, R.J., 2013. Oxidative stress and ageing of the post-ovulatory oocyte. Reproduction 146, 217-227. https://doi.org/10. 1530/REP-13-0111

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*. Biol. Reprod. 88, 1-9. http://dx.doi.org/10.1095/biolreprod.112.106450

Lyman-Gingerich, J., Pelegri, F., 2007. Maternal Factors in Fish Oocytes and Embryonic Development. In: The Fish Oocyte: From Basic Studies to Biotechnological Applications (Babin PJ, Cerda` J, Lubzens E, Eds.). Springer, New York, NY, USA.

Ma, H., Weber, G.M., Hostuttler, M.A., Wei, H., Wang, L., Yao, J., 2015. MicroRNA expression profiles from eggs of different qualities associated with post-ovulatory ageing in rainbow trout (Oncorhynchus mykiss). BMC Genomics. 16, 201. https://doi.org/10. 1186/s12864-015-1400-0

Ma, W., Zhang, D., Hou, Y., Li, Y.H., Sun, Q.Y., Sun, X.F., Wang, W.H., 2005. Reduced expression of MAD2, BCL2, and MAP kinase activity in pig oocytes after *in vitro* aging are associated with defects in sister chromatid segregation during meiosis II and embryo fragmentation after activation. Biol. Reprod. 72, 373-383. http://dx.doi.org/10.1095/biolreprod.104.030999

Mohandas, J., Marshall, J.J., Duggin, G.G., Horvath, J.S., Tiller, D.J., 1984. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney - possible implications in analgesic nephropathy. Biochem. Pharmacol. 33, 1801-1807. https://doi.org/10.1016/0006-2952(84)90353-8

Mommens, M., Fernandes, J.M., Bizuayehu, T.T., Bolla, S.L., Johnston, I.A., Babiak, I., 2010. Maternal gene expression in Atlantic halibut (*Hippoglossus hippoglossus* L.) and its relation to egg quality. BMC Res. Notes. 3, 138. https://doi.org/10.1186/1756-0500-3-138

Nikolic, A., Volarevic, V., Armstrong, L., Lako, M., Stojkovic, M., 2016. Primordial germ cells: current knowledge and perspectives. Stem Cells International. 1741072. http://dx.doi.org/10.1155/2016/1741072

Niksirat, H., Sarvi, K., Mojazi Amiri, B., Hatef, A., 2007. Effects of storage duration and storage media on initial and post-eyeing mortality of stored ova of rainbow trout *Oncorhynchus mykiss*. Aquaculture 262, 528–531. http://dx.doi.org/10.1016/j.aquaculture.2006.10.031.

Nishikimi, M., Rao, N.A., Yagi, K., 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochemical and Biophysical Research Communications. 46, 849-854. https://doi.org/10.1016/S0006-291X(72)80218-3

Pan, H., O'Brien, M.J., Wigglesworth, K., Eppig, J.J., Schultz, R.M., 2005. Transcript profiling during mouse oocyte development and the effect of gonadotropin priming and development *in vitro*. Dev. Biol. 286, 493-506. http://dx.doi.org/10.1016/j.ydbio.2005.08.023

Paynton, B.V., Bachvarova, R., 1994. Polyadenylation and deadenylation of maternal mRNAs during oocyte growth and maturation in the mouse. Mol. Reprod. Dev. 37, 172-180. http://dx.doi.org/10.1002/mrd.1080370208

Paynton, B.V., Rempel, R., Bachvarova, R., 1988. Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. Dev. Biol. 129, 304-314. https://doi.org/10. 1016/0012-1606(88)90377-6

Pechenino, A.S., Brown, T.R., 2006. Superoxide dismutase in the prostate lobes of aging Brown Norway rats. Prostate. 66, 522-35. http://dx.doi.org/10.1002/pros.20364

Raz, E., 2000. The function and regulation of vasa-like genes in germ-cell development. Genome Biology. 1(3). reviews1017

Rizzo, E., Godinho, H.P., Sato, Y., 2003. Short-term storage of oocytes from the neotropical teleost fish *Prochilodus marggravii*. Theriogenology 60, 1059–1070. http://dx.doi.org/10.1016/S0093-691X(03)00108-0.

Saffman, E., Lasko, P., 1999. Germline development in vertebrates and invertebrates. Cellul. Mol. Life. Sci. 55, 1141-1163. PMID: 10442094

Samarin, A.M., Sampels, S., Policar, T., Rodina, M., Hematyar, N., Samarin, A.M., 2018. mRNA abundance changes during *in vitro* oocyte ageing in African catfish *Clarias gariepinus* (Burchell, 1822). Aquac. Res. 49, 1037-1045. https://doi.org/10.1111/are.13552

Samarin, A.M., Blecha, M., Uzhytchak, M., Bytyutskyy, D., Żarski, 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. https://doi.org/10.1016/j.aquaculture.2015.08.017

Samarin, A.M., Policar, T., Lahnsteiner, F., 2015. Fish oocyte ageing and its effect on egg quality. Rev. Fish. Sci. Aquac. 23, 302-314. https://doi.org/10.1080/23308249.2015.1053560

Samarin, A.M., Żarski, D., Palińska-Żarska, K., Krejszeff, S., Blecha, M., Kucharczyk, D., Policar, T., 2017. *In vitro* storage of unfertilized eggs of the Eurasian perch and its effect on egg viability rates and the occurrence of larval malformations. Animal. 11(1), 78-83. https://doi.org/10.1017/S1751731116001361

Samarin, A.M., Mojazi Amiri, B., Soltani, M., Nazari, R.M., Kamali, A., Naghavi, M.R., 2011a. Effects of post-ovulatory oocyte ageing and temperature on egg quality in kutum Rutilus frisii kutum. World Appl. Sci. J. 15 (1), 14–18.

Smith, P.K., Krohn, R.I, Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76-85. https://doi.org/10.1016/0003-2697(85)90442-7

Steuerwald, N.M., Bermudez, M.G., Wells, D., Munne, S., Cohen, J., 2007. Maternal age-related differential global expression profiles observed in human oocytes. Reprod. Biomed. Online. 14, 700-708. https://doi.org/10.1016/S1472-6483(10)60671-2

Sturtz, L.A., Diekert, K., Jensen, L.T., Lill, R., Culotta, V.C., 2001. A fraction of yeast Cu, Zn superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria: a physiological role for SOD1 in guarding against mitochondrial oxidative damage. J Biol Chem. 276, 38084-9. http://dx.doi.org/10.1074/jbc.M105296200

Tadros, W., Lipshitz, H.D., 2009. The maternal-to-zygotic transition: A play in two acts. Development 136, 3033-3042. https://doi.org/10. 1242/dev.033183

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. Biol. Reprod. 80, 493–502. https://doi.org/10.1095/biolreprod.108.072017

Takahashi, T., Saito, H., Hiroi, M., Doi, K., Takahashi, E., 2000. Effects of aging on inositol 1,4,5-triphosphate-induced Ca(2 +) release in unfertilized mouse oocytes. Mol. Reprod. Dev. 55, 299-306. https://doi.org/10.1002/(ISSN)1098-2795

Takahashi, T., Takahashi, E., Igarashi, H., Tezuka, N., Kurachi, H., 2003. Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. Mol. Reprod. Dev. 66, 143-152. https://doi.org/10.1002/(ISSN)1098-2795

Tarin, J.J., Perez-Albala, S., Cano, A., 2000. Consequences on offspring of abnormal function in ageing gametes. Human Reproduction Update. 6, 532-549. https://doi.org/10.1093/humupd/6.6.532

Tarin, J.J., Perez-Albala, S., Perez-Hoyos, P., Cano, A., 2002. Postovulatory aging of oocytes decreases reproductive fitness and longevity of offspring. Biol. Reprod. 66, 495-499. https://doi.org/10. 1095/biolreprod66.2.495

Tzung, K.W., Goto, R., Saju, J.M., Sreenivasan, R., Saito, T., Arai, K., Yamaha, E., Hossain, M.S., Calvert, M.E.K., Orban, L., 2015. Early Depletion of Primordial Germ Cells in Zebrafish Promotes Testis Formation. Stem. Cell. Rep. 4(1), 61-73. https://doi.org/10.1016/j.stemcr.2014.10.011

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3 - new capabilities and interfaces. Nucleic Acids Research. 40(15), e115. https://doi.org/10. 1093/nar/gks596

Verbeke, P., Fonager, J., Clark, B.F., Rattan, S.I., 2001. Heat shock response and ageing: mechanisms and applications. Cell. Biol. Int. 25, 845-857. http://dx.doi.org/10.1006/cbir.2001.0789

Weill, L., Belloc, E., Bava, F.A., Mendez, R., 2012. Translational control by changes in poly(A) tail length: Recycling mRNAs. Nat. Struct. Mol. Biol. 19, 577-585. https://doi.org/10.1038/nsmb.2311

Wesson, D.E., Elliott, S.J., 1995. The H2O2-generating enzyme, xanthine oxidase, decreases luminal Ca2+ content of the IP3- sensitive Ca2+ store in vascular endothelial cells. Microcirculation 2, 195-203. PMID: 7497171

Xu, Z., Abbott, A., Kopf, G.S., Schultz, R.M., Ducibella, T., 1997. Spontaneous activation of ovulated mouse eggs: time-dependent effects on M-phase exit, cortical granule exocytosis, maternal messenger ribonucleic acid recruitment, and inositol 1,4,5-triphosphate sensitivity. Biol Reprod. 57, 743-750. PMID: 9314575

Zuccotti, M., Merico, V., Cecconi, S., Redi, C.A., Garagna, S., 2011. What does it take to make a developmentally competent mammalian egg? Hum Reprod Update. 17, 525-540. http://dx.doi.org/10.1093/humupd/dmr009

CHAPTER 6

GENERAL DISCUSSION

As time elapses after ovulation, oocytes experience changes that negatively affect the egg quality and successive developmental stages. The optimum time of fertilization after ovulation or stripping varies from a few minutes to a few weeks depending on the fish species and the storage temperature (Samarin et al. 2015b). Regardless of the storage temperature, both the embryo mortality and larval malformation rates increased significantly over time and were the highest in the most aged oocytes in African catfish and common carp. The observed decreasing trend for the embryo survival, eyeing and hatching rates and the increasing trend for the embryo mortality and larval malformation percentages after the ovulation in our studies are in accordance with the previously performed experiments on other fish species (*e.g.*, Lahnsteiner et al. 2001; Aegerter et al. 2004; Samarin et al. 2016). Lower egg storage temperatures usually guarantee longer successful storage time compared to higher temperatures (e.g., Azuma et al. 2003; Samarin et al. 2008, 2017). According to our knowledge and previous works of our research group working with both *in vivo* and *in vitro* egg storage in several fish species, the variability of egg quality through oocyte ageing among individual females is very different depending on the fish species. For instance, the standard deviation for all measured viability parameters in rainbow trout (*Oncorhynchus mykiss*) (Samarin et al. 2008) and common carp (*Cyprinus carpio*) (Samarin et al. 2015b), which are more domesticated species, is very low, while for northern pike (*Esox Lucius*) (Samarin et al. 2016) and Eurasian perch (*Perca fluviatilis*) (Samarin et al. 2017), which are more wild species, the standard deviation is very high. The effect of domestication has not yet been studied on the progress of oocyte ageing.

The precise molecular mechanisms underlying post-ovulatory oocyte ageing and its deleterious effects are still unclear in fish and in other vertebrates. Most findings in this field have been obtained from studies on other vertebrates. It has not yet been clearly determined whether the oocyte ageing process is started by an initiating factor and is followed by a series of events that finally lead to loss of the fertilizing ability or whether different cellular and molecular events start separately and then join each other to complete the process. Some authors believe that increasing ROS and the subsequent oxidative stress may act as an initiating factor for the deleterious effects of oocyte ageing (Lord and Aitken, 2013; Takahashi et al. 2013; Lord et al. 2013). The intra-cytoplasmic level of glutathione (GSH), which has a major role in protecting oocytes from damage by ROS, decreased in aged mouse oocytes (Boerjan and de Boer, 1990). Additionally, the level of lipid peroxidation, which is an indicator of the degree of oxidative stress, increased in *in vivo* aged mouse oocytes (Takahashi et al. 2003). In human embryos, a positive correlation has been reported between the concentration of hydrogen peroxide and apoptosis occurrence (Yang et al. 1998), indicating the initiating role of hydrogen peroxide on ageing. Lord et al. (2013) suggested that increased oxidative stress can be considered an early marker of oocyte ageing before the activation of caspases and before the appearance of the morphological features of oocyte ageing and apoptosis. To delay or even prevent the ageing process in oocytes stored *in vitro*, treating them with some chemicals and antioxidant agents, such as caffeine in pigs (Kikuchi et al. 2002), nitric oxide (Goud et al. 2005) and DL-dithiothreitol in mice (Rausel et al. 2007) and humans (Tarin et al. 1998) and trichostatin A in pigs (Jeseta et al. 2008) and mice (Huang et al. 2007), has been proposed and applied. Additionally, melatonin has been reported to reduce oxidative stress in aged mouse oocytes and to delay the onset of apoptosis, which is the end point of the oocyte ageing process (Lord et al. 2013).

Hence, we examined whether oxidative stress affected the progress of oocyte ageing, as has been proposed for other vertebrates. We reported some molecular changes during oocyte ageing in African catfish, goldfish and common carp. Our results demonstrated no significant

changes in the mRNA levels of oxidative stress-related genes and the genes involved in cell cycling during the progress of oocyte ageing in any of the examined species. Additionally, with elapsing time following ovulation, the amount of TBARs, which is the main indicator of lipid peroxidation, and the amount of carbonyls, which show the extension of protein oxidation, did not change in any of the oocytes from our examined species. Enzymatic antioxidant systems can scavenge ROS and, therefore, decrease the effect of oxidative stress. If oxidative stress is the initiator of deleterious effects during post-ovulatory oocyte ageing, then an alteration of antioxidant enzyme activity and oxidation markers should occur following ovulation. Our results indicated no significant changes in the activity of CAT and SOD during the post-ovulatory ageing of common carp, goldfish and tench oocytes. However, antioxidant enzyme analysis showed a significant decrease in the activity of GPX during the post-ovulatory ageing of tench oocytes, which could play a major role in the overall drop in egg quality. In fact, the egg quality indicator (embryo survival) in the case of the tench, started to decrease from 6 hours post-stripping, while the GPX activity significantly decreased at 8 hours. This kind of consequence of the ageing process was not observed in common carp and goldfish oocytes.

In addition to the direct effects of oxidative stress on oocytes, changes of fatty acid and lipid composition caused by oxidation could also affect the functionality. For example, lipid peroxidation can cause changes in the membrane fluidity that might subsequently cause decreased fusion of sperm and oolemma and decreased fertilization rate (Lord and Aitken, 2013). In this respect, Mansour et al. (2011) have evaluated changes in fatty acid composition in relation to the fertility of fish eggs and found some significant differences between eggs with high and low fertility rates. More recently, Mok et al. (2016) also reported that several phospholipid classes, including phosphatidic acid, phosphatidylinositol, phosphatidylserine, and lysophosphatidylserine, significantly decreased both in H_2O_2-treated and aged oocytes. Moreover, there has been some indication that changes in fatty acid and lipid composition are related to egg quality during ageing. For example, in rainbow trout over-ripening was accompanied by an increase in free lipids and decrease in lipid phosphorus as a measure for phospholipids (Craik and Harvey, 1984). The levels of esterified and non-esterified fatty acids were also significantly decreased in over-ripened rainbow trout eggs (Lahnsteiner, 2000). In Caspian brown trout, the level of triglycerides decreased 30 days post-ovulation (Bahrekazemi et al. 2010). Regarding mitochondrial dysfunction in the over-ripened oocytes, changes in phospholipid composition due to oxidative stress might play an important role. In mitochondria especially, the phospholipid groups cardiolipin and sphingomyelin are important for membrane functionality. Both cardiolipin and sphingomyelin are especially sensitive to oxidation due to their high content of polyunsaturated fatty acids (Paradies et al. 2009). These authors suggested that any change in cardiolipin structure would result in mitochondrial dysfunction. It has already 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). Therefore, according to our obtained results, oxidative stress is not the main initiator or promotor of the oocyte ageing process. However, complementary tests and analysis are required to clearly clarify its involvement.

The apoptotic related factors, however, showed an increase in their mRNA level during the oocyte ageing. Apoptotic cell death is the end point of the oocyte ageing process and has been reported to occur through caspase activation (Takai et al. 2007), increased levels of the apoptotic signalling protein Bax, decreased levels of the anti-apoptotic protein Bcl2 (Perez et al. 2005) and DNA damage (Fujino et al. 1996). We observed that the African catfish oocyte ageing resulted in an upward trend in the mRNA level of cathepsin D, an apoptosis-related gene. Aegerter et al. (2005) also found that *in vivo* oocyte ageing in rainbow trout is associated with the increased mRNA levels of cathepsin Z. Lysosomal proteases, such as

cathepsin D and cathepsin B, act as pro-apoptotic mediators (Kagedal et al. 2011). We found that the mRNA abundance of transcripts involved in apoptosis, such as *caspase3A*, *caspase9* and *bax*, are higher in over-ripened eggs than those at the time of ovulation in common carp both in *in vivo* and *in vitro* conditions. By contrast, it has been shown that the levels of *bax* remains unchanged in the mouse oocytes aged *in vitro*. (Gordo et al. 2002; Takahashi et al. 2013). At the end of the oocyte ageing time, pro-apoptotic molecules such as *bax* induce the release of *cytochrome c*, which activates caspases, while anti-apoptotic molecules such as *bcl2* prevent this release (Li et al. 2000). Our preliminary results with the zebrafish *Danio rerio* (vasa GFP transgenic strain), indicated that oocyte ageing significantly affects the number and development of the primordial germ cells (PGCs). Since depletion of PGCs converts the sex differentiation in favour of males in zebrafish (Tzung et al. 2015) and other fish species (liu et al. 2015), oocyte ageing may bias the sex ratio in favour of males or the occurrence of completely sterile individuals. This idea needs to be addressed in future studies.

Further analysis of these genes during development in eggs at varying ageing times will be useful and will benefit the study of fish egg quality. Although examining the mRNA abundance of single target genes could be a good and helpful tool, it is not yet enough to make conclusions on the possible involvement of oxidative stress in the progress of fish oocyte ageing. In fact, additional analysis, such as microarray analysis, total ROS measurement, mitochondrial dysfunction indicators, ATP content of the eggs, etc., are required to fully evaluate the contribution of oxidative stress to the drop of egg quality during postovulatory ageing. Additionally, as there is no clear link between mRNA abundance and protein synthesis in metaphase 2 oocytes, studying the proteome profile changes during fish oocyte ageing could provide valuable information about the oocyte ageing and its underlying mechanisms.

CONCLUSIONS

Unfertilized ovulated oocytes contain maternal mRNAs that direct the early stages of embryogenesis until the zygotic transcription is activated. Several studies have demonstrated that oocyte ageing is associated with the accumulation of molecular damage, including altered relative mRNA abundance in the eggs of fish and other animals. Identifying mRNAs and proteins that are differentially expressed during fish oocyte ageing and discovering the underlying mechanisms of gene and protein regulation would contribute information that is valuable to basic research and for practical applications in aquaculture. No study has yet been performed on the effect of fish oocyte ageing on the subsequent growth and genetic structure of progeny. This topic should also be addressed in future studies. In addition, almost nothing is known of "maternal oocyte ageing" in fishes, which might be an excellent model for studying the phenomenon.

REFERENCES

Aegerter, S., Jalabert, B. and Bobe, J. (2004) MessengerRNAstockpile of cyclin B, insulin-like growth factor I, insulin-like growth factor II, insulinlike growth factor receptor Ib, and p53 in the rainbow trout oocyte in relation with developmental competence. *Mol Reprod Dev* 67, 127–135.

Aegerter, S., Jalabert, B. and Bobe, J. (2005). Large scale real-time PCR analysis of mRNA abundance in rainbow trout eggs in relationship with egg quality and post-ovulatory ageing. *Molecular Reproduction and Development* 72, 377–385.

Azuma, T., H. Ohta, S. Oda, K. Muto, T. Yada, and T. Unuma. Changes in fertility of rainbow trout eggs retained in coelom. Fish. Sci., 69: 131–136 (2003).

Bahrekazemi, M., Soltani, M., Matinfar, A., Abtahi, B., Pusti, I., Samarin, A.M. and Mojazi Amiri, B. (2010) Biochemical and histological studies of over-ripened oocyte in the Caspian brown trout (Salmo trutta caspius) to determine biomarkers for egg quality. *Iranian Journal of Fisheries Sciences* 9, 33–48.

Boerjan ML, de Boer P. 1990. First cell cycle of zygotes of the mouse derived from oocytes aged postovulation *in vivo* and fertilized *in vivo*. Mol Reprod Dev 25:155–163.

Craik, J.C.A. & Harvey, S.M. (1984) Biochemical changes associated with overripening of the eggs of rainbow trout, *Salmo gairdneri*. *Aquaculture* 37, 347-357.

Fujino, Y., Ozaki, K., Yamamasu, S., Ito, F., Matsuoka, I., Hayashi, E., Nakamura, H., Ogita, S., Sato, E. and Inoue, M. (1996) DNA fragmentation of oocytes in aged mice. *Human Reproduction* 11, 1480-1483.

Gordo, A.C., Rodrigues, P. and Kurokawa, M. (2002) Intracellular calcium oscillations signal apoptosis rather than activation in *in vitro* aged mouse eggs. *Biol. Reprod* 66, 1828–1837.

Goud AP, Goud PT, Diamond MP, Abu-Soud HM. Nitric oxide delays oocyte aging. Biochemistry. 2005; 44:11361–11368.

Huang, J. C., L.Y. Yan, Z. L. Lei, Y. L. Miao, L. H. Shi, J. W. Yang, Q. Wang, Y. C. Ouyang, Q. Y. Sun, D. Y. Chen. (2007) Changes in histone acetylation during postovulatory aging of mouse oocyte. *Biol. Reprod* 77, 666–670.

Jeseta M, Petr J, Krejcova T, Chmelikova E, Jilek F (2008) In vitro ageing of pig oocytes: effects of the histone deacetylase inhibitor trichostatin A. Zygote 16: 145–152.

Kagedal, K., Johansson, U., & € Ollinger, K. (2001). The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. Faseb Journal, 15, 1592–1594.

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 and Stem Cells* 4: 211–222.

Lahnsteiner, F. (2000) Morphological, physiological and biochemical parameters characterizing the overripening of rainbow trout eggs. *Fish Physiology and Biochemistry* 23, 107-118.

Lahnsteiner, F., B. Urbanyi, A. Horvath, and T. Weismann. Biomarkers for egg quality determination in cyprinid fish. Aquaculture, 195: 331–352 (2001)

Li, M., Baumeister, P., Roy, B., Phan, T., Foti, D., Luo, S. and Lee, A.S. (2000) ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. *Mol Cell Biol* 20, 5096–5106.

Lord T, Aitken RJ (2013) Oxidative stress and ageing of the post-ovulatory oocyte. *Reproduction* 146: 217–227.

Lord T, Nixon B, Jones KT, Aitken RJ (2013) Melatonin prevents postovulatory oocyte aging in the mouse and extends the window for optimal fertilization *in vitro*. *Biology of Reproduction* 88: 1–9.

Mansour, N., Lahnsteiner, F., Mcniven, M. A., Richardson, G. F. & Pelletier, C. S. (2011) Relationship between fertility and fatty acid profile of sperm and eggs in Arctic char, *Salvelinus alpinus*. *Aquaculture* 318, 371-378.

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, e0148577.

Paradies, G., Petrosillo, G., Paradies, V., Ruggiero, F. M. Role of cardiolipin peroxidation and Ca2+ in mitochondrial dysfunction and disease. Cell Calcium, 45, 643-650 (2009).

Perez, G.I., Juriscova, A., Matikainen, T., Morityama, T., Kim, M.R., Takai, Y., Pru, J.K., Kolesnick, R.N. and Tilly, J.L. (2005) A central role for ceramide in the age-related acceleration of apoptosis in the female germline. *Faseb Journal* 19, 860-862.

Rausell, F., J. F. Pertusa, V. Gomez-Piquer, C. Hermenegildo, M. A. Garcia-Perez, A. Cano, and J. J. Tarin. Beneficial effects of dithiothreitol on relative levels of glutathione S-transferase activity and thiols in oocytes, and cell number, DNA fragmentation and allocation at the blastocyst stage in the mouse. Mol. Reprod. Dev., 74: 860–869 (2007).

Samarin AM, Blecha M, Uzhytchak M, Bytyutskyy D, Żarski 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 AM, Żarski D, Palińska-Żarska K, Krejszeff S, Blecha M, Kucharczyk D, Policar T, 2017: *In vitro* storage of unfertilized eggs of the Eurasian perch and its effect on egg viability rates and the occurrence of larval malformations. Animal (first view) 1-6.

Samarin, A. M., Gela, D., Bytyutskyy, D., Policar, T., 2015b. Determination of the best post-ovulatory stripping time for the common carp, *Cyprinus carpio*. Journal of Applied Ichthyology, 31 (Suppl. 2): 51-55.

Samarin, A. M., M. R. Ahmadi, T. Azuma, Gh. R. Rafiee, B. Mojazi Amiri, and M. R. Naghavi. Influence of the time to egg stripping on eyeing and hatching rates in rainbow trout Oncorhynchus mykiss under cold temperatures. Aquaculture, 278: 195–198 (2008).

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. *The Journal of Obstetrics and Gynaecology Research* 39: 1431–1439.

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* 66: 143–152.

Takai Y, Matikainen T, Juriscova A, Kim MR, Trbovich AM, Fujita E, Nakagawa T, Lemmers B, Flavell RA, Hakem R, Momoi T, Yuan J, Tilly JL, Perez GI. Caspase-12 compensates for lack of caspase-2 and caspase-3 in female germ cells. Apoptosis 2007; 12: 791-800.

Tarin JJ, Vendrell FJ, Ten J, Cano A (1998) Antioxidant therapy counteracts the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes. Molecular Human Reproduction 4: 281–288.

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum Reprod. 1998; 13:998–1002.

ENGLISH SUMMARY

Alteration of mRNA abundance, oxidation products and antioxidant enzyme activities associated with fish oocyte ageing

Azadeh Mohagheghi Samarin

In fish, matured eggs are released from follicle cells into the ovarian or body cavity during ovulation while they are in the second meiotic division stage of metaphase, and they remain there until spawning is stimulated by environmental factors or eggs are collected by artificial techniques. Delayed spawning in nature, delayed egg collection in capture and delayed fertilization after egg stripping lead to excessive oocyte ageing and, finally, over-ripening. During over-ripening, morphological, physiological, biochemical, histological, cellular and molecular changes occur inside the eggs that negatively affect the egg fertilization capacity and successive developmental stages. Until now, there has been only a poor understanding about the processes and underlying mechanisms of oocyte ageing in fish as well as in other vertebrates. Some studies on other vertebrates have suggested that oxidative stress is the initiating factor for the progress of oocyte ageing. Alterations of the lipidome associated with oocyte ageing reported that several phospholipid classes were significantly decreased in both H_2O_2-treated and aged oocytes, suggesting the involvement of oxidative stress in lipid plasma membrane composition and, as a result, unfavourable outcomes in oocyte ageing.

It is possible that some egg quality defects are induced through abnormal mRNA abundance in the oocyte. Therefore, we examined changes in the mRNA levels of transcripts associated with oocyte ageing in fish. The present work investigated possible changes in the mRNA levels of transcripts involved in oxidative damage, mitochondrial function and stress response, with roles in fertilization, embryo development, transcriptional regulation and the cell cycle as well as others related to apoptosis during egg over-ripening. This study investigated the oxidation status of oocytes during post-ovulatory ageing by measuring TBARs as a marker of lipid oxidation and carbonyls, which show the extent of protein oxidation. In addition, the role of oxidative stress in the progress of oocyte ageing was assessed by evaluating the activity of antioxidant enzymes, including CAT, SOD, GPX and GR. Possible changes in lipid class and fatty acid composition during fish oocyte ageing were also examined. Egg viability parameters and larval ploidy levels were examined.

A complete loss of egg viability for African catfish (*Clarias gariepinus*) occurred at 16 and 24 hours post-stripping (HPS) when eggs were stored at 25°C and 4°C, respectively. Under both storage temperatures, the embryo mortality and larval malformation rates increased significantly over time and were the highest in the most aged oocytes. We determined the time in which the eggs retain their fertilizing ability to be 10 hours for tench (*Tinca tinca*) at 20 °C, 18 hours for goldfish (*Carassius auratus*) at 20 °C and 14 hours post-ovulation (HPO) and more than 10 hours post-stripping (HPS) for common carp (*Cyprinus carpio*) at 20 °C. Our results demonstrated no significant changes in the mRNA levels of oxidative stress related genes and genes involved in the cell cycling during the progress of oocyte ageing in any of species, including African catfish, goldfish or common carp. Additionally, with elapsing time following ovulation, the amount of TBARs and carbonyls did not change in any of the oocytes from our experimental species, including African catfish, tench, goldfish and common carp. However, an increase in the mRNA abundance of apoptotic related genes was observed. Antioxidant enzyme analysis indicated no significant changes in the activity of CAT and SOD during the post-ovulatory ageing of common carp, goldfish and tench oocytes. However, a significant decrease in the activity of GPX was observed during the post-ovulatory

ageing of tench oocytes, which could play a major role in the overall drop of egg quality. This observation of decreased GPX activity was not observed in common carp and goldfish oocytes. In addition to the direct effects of oxidative stress on oocytes, changes of fatty acid and lipid composition caused by oxidation could also affect the functionality. We observed that post-ovulatory ageing of the oocytes does not change the fatty acid and lipid class composition of the oocytes. Therefore, according to our obtained results, oxidative stress is not the main initiator or promotor of the oocyte ageing process. However, complementary tests and analysis are required to clearly clarify its involvement. Increased mRNA levels of apoptotic related transcripts during oocyte ageing in this study demonstrate that the apoptotic pathway might be involved in the molecular changes during the progress of oocyte ageing. Investigation of epigenetic changes associated with fish oocyte ageing appears to be of interest for future research work.

CZECH SUMMARY

Změny v množství mRNA, v aktivitě oxidativní produktů a antioxidačních enzymů v závislosti na stárnutí rybích oocytů

Azadeh Mohagheghi Samarin

V průběhu ovulace jsou zralé oocyty ve stadiu metafáze druhého meiotického dělení uvolněny z folikulů do ovárií nebo břišní dutiny a zůstávají zde až do okamžiku samotného výtěru, který buď probíhá přirozeně, nebo je proveden uměle. Opožděná přirozená reprodukce či umělý výtěr oocytů, a také jejich pozdější oplodnění při umělém výtěru vede k jejich stárnutí a následně přezrávání. V průběhu procesu přezrávání oocytů dochází k různým morfologickým, fyziologickým, biochemickým, histologickým, buněčným a molekulárním změnám, které následně negativně ovlivňují oplozeníschopnost oocytů a také vývoj embryí. I v dnešní době jsou ovšem znalosti o procesu stárnutí oocytů ryb nebo vyšších obratlovců velmi omezené. Některé studie prováděné na vyšších obratlovcích předpokládají, že na stárnutím oocytů má největší vliv zejména oxidativní stres. Změny lipidů (pokles v několika skupinách fosfolipidů, jak u oocytů ošetřených peroxidem vodíku, tak u starších oocytů) při stárnutí oocytů ukazují na fakt, že oxidativní stres má negativní vliv na složení lipidové plazmatické membrány.

V některých případech může být ale špatná kvalita jiker způsobena abnormálním množstvím mRNA v oocytu. Z tohoto důvodu jsme zkoumali změny v mRNA transkriptu (přepisu), které jsou spojené se stárnutím oocytů. Tato práce se zabývá možnými změnami v mRNA transkriptu, který je způsobený oxidativním poškozením buněk, mitochondriální funkcí a stresovou reakcí a souvisí s následnou oplozeníschopností oocytů, vývojem embryí, transkripční regulací a cyklem buněk, a také apoptózou během přezrávání oocytů. Tato práce také sleduje oxidativní stavy oocytů během post-ovulačního stárnutí pomocí hodnot TBAR jakožto ukazatele oxidace lipidů a karbonylů ukazujících na prodloužení oxidace proteinů. Dále byla role oxidativního stresu v průběhu stárnutí oocytů hodnocena pomocí sledování aktivity antioxidačních enzymů jako jsou: CAT, SOD, GPX a GR. Dalšími hodnocenými parametry byly změny v jednotlivých skupinách lipidů, mastných kyselin, životaschopnosti oocytů a ploidní úroveni vylíhnutých larev. Důvodem proč byly vybrány ryby jako modelový organizmus je jejich velká rozmanitost v reprodukční biologii mezi jednotlivými druhy a možnost získat velké množství oocytů od jednoho jedince v porovnání s ostatními živočichy. Protože se setkáváme se stále rostoucím zájmem o znalosti v oblasti asistované reprodukce, je studium stárnutí oocytů z praktického pohledu velmi důležité.

V případě sumečka afrického (*Clarias gariepinus*) dochází k přezrání oocytů 16 hodin po výtěru při jejich uchovávání v teplotě 25 °C a za 24 hodin po výtěru u jiker skladovaných při 4 °C. V případě obou sledovaných teplot bylo pozorováno postupné zvyšování mortality a počtu deformovaných larev v průběhu jejich uchovávání. Nejvyšší mortalita a nejnižší kvalita larev byly zaznamenány u nejdéle uchovávaných oocytů. Dále byl u vybraných druhů ryb stanoven čas, po který si jejich oocyty stále zachovávaly vysokou oplozeníschopnost. U lína obecného (*Tinca tinca*) je to 10 hodin při teplotě 20 °C, u karase zlatého (*Carassius auratus*) 18 hodin při 20 °C a v případě kapra obecného (*Cyprinus carpio*) je to 14 hodin po ovulaci a 10 hodin po umělém výtěru při 20 °C. Z výsledků práce je patrné, že v průběhu stárnutí oocytů nedošlo k žádné průkazné změně mRNA genů týkajících se oxidativního stresu a genů zapojených do buněčného cyklu během procesu stárnutí oocytů u sumečka afrického, karase zlatého a kapra obecného. Navíc u sledovaných druhů nedošlo v průběhu stárnutí oocytů ke změně hodnot TBAR a karbonylů. Avšak bylo pozorováno zvýšené množství mRNA apopticky příbuzných genů. Analýza antioxidačních enzymů nezaznamenala významné změny

v hodnotách CAT a SOD během stárnutí oocytů po jejich získání u kapra obecného, karase zlatého a lína obecného. Nicméně významně snížená aktivita GPX byla pozorována při stárnutí oocytů u lína obecného, která by mohla odhalovat její významou roli v celkovém poklesu kvality jiker. Zmíněná snížená aktivita GPX nebyla pozorována u oocytů kapra obecného a karase zlatého. Kromě přímých účinků oxidativního stresu na oocyty by mohly jejich funkčnost ovlivnit i změny kompozice mastných kyselin a lipidů, které jsou způsobené oxidací. Pozorovali jsme, že při stárnutí oocytů se nemění složení mastných kyselin a skupin lipidů v oocytech. Proto podle našich získaných výsledků není oxidativní stres hlavním iniciátorem nebo promotorem procesu stárnutí oocytů. Avšak komplementární testy a analýzy jsou potřebné k jasnému vysvětlení jeho vlivu a zapojení do celého procesu stárnutí oocytů. Zvýšené hladiny mRNA apoptoticky souvisejících transkriptů během stárnutí oocytů v této práci demonstrují, že apoptotická dráha může být zapojena do molekulárních změn během stárnutí oocytů. Studium epigenetických změn spojených se stárnutím rybích oocytů se jeví jako velmi zajímavý výzkumný směr pro budoucí vědecké studie.

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LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Samarin, A.M., Sampels, S., Krzyskow, A., Burkina, V., Křišťan, J., Gela, D., Flajšhans, M., Policar, T., Samarin, A.M., 2018. 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, 2305–2316. (IF 2017 = 1.475)

Samarin, A.M., **Samarin, A.M.,** Policar, T., 2018. Cellular and molecular changes associated with fish oocyte ageing. Reviews in Aquaculture 1–12, doi: 10.1111/raq.12249. (IF 2017 = 7.139)

Samarin, A.M., Sampels, S., Policar, T., Rodina, M., Hematyar, N., Mohagheghi Samarin, A., 2018. mRNA abundance changes during *in vitro* oocyte ageing in African catfish *Clarias gariepinus* (Burchel, 1822). Aquaculture Research 49, 1037–1045. (IF 2017 = 1.475)

Reichetzeder, C., von Websky, K., Tsuprykov, O., **Samarin, A.M.,** Falke, L.G., Putra, S.E.D., Hasan, A.A., Antonenko, V., Curato, C., Rippmann, J., Klein, T., Hocher, B., 2017. Head-to-head comparison of structurally unrelated dipeptidyl peptidase 4 inhibitors in the setting of renal ischemia reperfusion injury. British Journal of Pharmacology 174, 2273–2286. (IF 2016 = 5.491)

Peer-reviewed journals without IF

Dezashibi, Z., **Samarin, A.M.,** Hematyar, N., Haddad Khodaparast, M., 2013. "Phenolics in Henna: Extraction and Stability". European Journal of Experimental Biology 3, 38–41.

Hematyar, N., **Samarin, A.M.,** Poorazarang, H., Elhamirad, A., 2012. "Effect of gums on yogurt characteristics". World Applied Sciences Journal 20, 661–665.

Samarin, A.M., Poorazarang, H., Hematyar, N., Elhamirad, A. 2012. "Phenolics in Potato Peels: Extraction and Utilization as Natural Antioxidants". World Applied Sciences Journal 18, 191–195.

Samarin, A.M., Poorazarang, H., Akhlaghi, H., Elhami Rad, A., Hematyar, N., 2008. Antioxidant activity of potato (*Solanum tuberosum*, raja) peel extract. Iranian Journal of Nutrition Sciences and Food Technology 3, 23–32.

Abstracts and conference proceedings

Samarin, A.M., Sampels, S., Policar, T., Rodina, M., Hematyar, N., Samarin, A.M., 2017. mRNA expression changes during *in vitro* oocyte ageing in African catfish *Clarias gariepinus*. Aquaculture Europe 2017, Dubrovnik, Croatia, October 17–20, 2017. (Poster presentation)

Samarin, A.M., Sampels, S., Krzyskow, A., Østbyec, T.-K.K., Blecha, M., Kristan, J., Gela, D., Flajshans, M., Policar, T., Samarin. A.M., 2017. Lipid classes and fatty acid composition profile associated with *in vitro* oocyte ageing in tench (*Tinca tinca*). 6th International Workshop on the Biology of Fish Gametes, Ceske Budejovice, Czech Republic, September 4–7, 2017. (Poster presentation)

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Samarin,A.M.,Samarin, A.M., Policar, T., Blecha, M., Ruyter, B., Østbye, TK., Andersen, Ø., Sampels, S., 2016. Alteration of mRNA expression patterns associated with post-stripping oocyte ageing in goldfish *Carassius auratus.* 8th International Symposium on Fish Endocrinology, Gothenburg, Sweden, June 28th–July 2nd, 2016. (Poster presentation)

Reichetzeder, C., Antonenko, V., Hocher, B., **Samarin,A.M.,** Tsuprykov, O., von Websky, K., 2015. Effects of DPP-4 inhibition on glomerular and tubular function in a rat model of ischaemia-reperfusion injury. Oral Abstract #70. EASD virtual meeting. Stockholm, Sweden, September, 2015. (Poster presentation)

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Gholi, N., Mashhad, S., Akhlaghi, H., Dezashibi, Z., **Mohagheghi Samarin, A.,** 2010. Effect of two extraction methods on the antioxidant activity of henna (*Lawsonia inermis*) leaves. The 6th CMAPSEEC, Antalya, Turkey, Apr–Jun, 2010, Pharmacognosy Magazine 6, p. 114. (Poster presentation)

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Samarin, A.M., Sampels, S., Krzyskow, A., Østbyec, T.-K.K., Blecha, M., Kristan, J., Gela, D., Flajshans, M., Policar, T., Samarin. A.M., 2017. Lipid classes and fatty acid composition profile associated with *in vitro* oocyte ageing in tench (*Tinca tinca*). 6th International Workshop on the Biology of Fish Gametes, Ceske Budejovice, Czech Republic, September 4–7, 2017. (Poster presentation)	2017
Samarin, A.M., **Samarin,A.M.**, Østbye, T.-K.K., Andersen, Ø., Ruyter, B., Sampels, S., Blecha, M., Gela, D., Policar, T., 2017. Changes in mRNA levels of transcripts associated with oocyte ageing Common Carp, *Cyprinus carpio*. 6th International Workshop on the Biology of Fish Gametes, Ceske Budejovice, Czech Republic, September 4–7, 2017. (Oral presentation)	2017
Samarin, A. M., **Samarin, A.,** Blecha, M., Kristan, J., Gela, D., Sampels, S., Flajshans, M., Østbye, TK., Policar, T., 2016. Egg RNA content and embryo survival rates associated with *in vitro* ova ageing in tench (*Tinca tinca* L.). FABA 2016: International Symposium on Fisheries and Aquatic Sciences. Antalya, Turkey, November 3–5, 2016. (Oral presentation)	2016
Samarin,A.M.,Samarin, A.M., Policar, T., Blecha, M., Ruyter, B., Østbye, TK., Andersen, Ø., Sampels, S., 2016. Alteration of mRNA expression patterns associated with post-stripping oocyte ageing in goldfish *Carassius auratus*. 8th International Symposium on Fish Endocrinology, Gothenburg, Sweden, June 28th–July 2nd, 2016. (Poster presentation)	2016
Reichetzeder, C., Antonenko, V., Hocher, B., **Samarin,A.M.,** Tsuprykov, O., von Websky, K., 2015. Effects of DPP-4 inhibition on glomerular and tubular function in a rat model of ischaemia-reperfusion injury. Oral Abstract #70. EASD virtual meeting. Stockholm, Sweden, September, 2015. (Poster presentation)	2015
Reichetzeder, C., von Websky, K., Tsuprykov, O., Antonenko, V., **Samarin,A.M.,** Klein, T., Hocher, B., 2015. Divergent effects of DPP-4 inhibition on glomerular and tubular function in a rat model of ischemia-reperfusion injury. American Diabetes Association 75th, Boston, MA, America, 5–9 June, 2015. (Oral presentation)	2015

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