Fakulta rybářství
a ochrany vod
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Jihočeská univerzita
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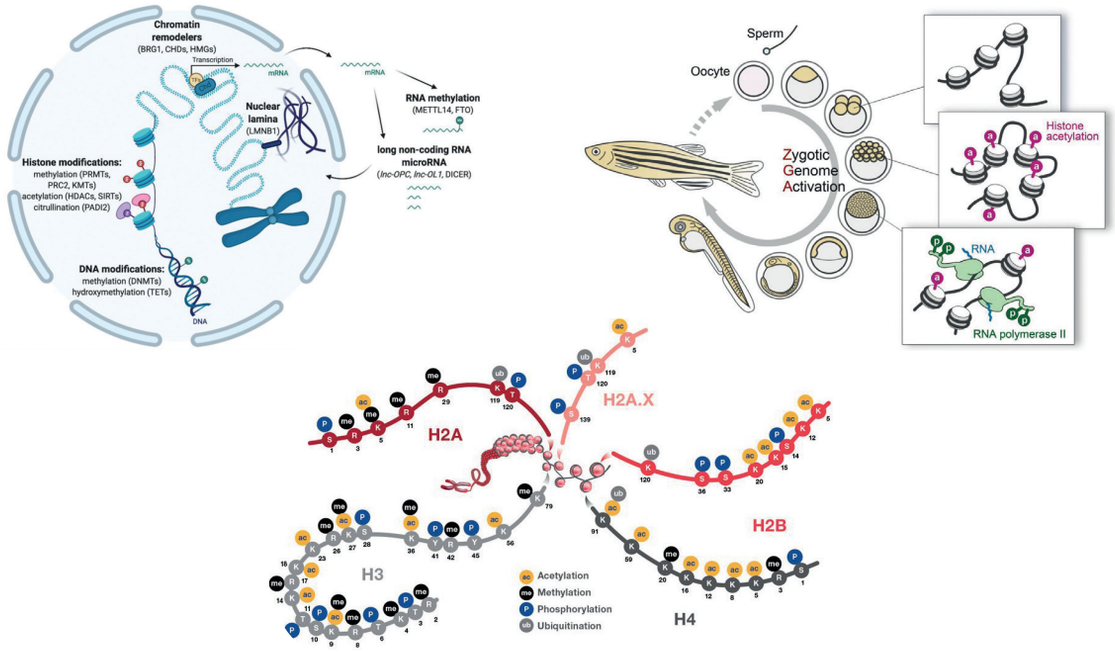
Fish oocyte ageing and histone modifications

Stárnutí rybích oocytů a modifikace histonů



Doctoral thesis by
Swapnil Gorakh Waghmare

Czech Republic, Vodňany, 2022

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Supervisor:
Azin Mohagheghi Samarin, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Consultant:
Azadeh Mohagheghi Samarin, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Head of Laboratory of Intensive Aquaculture:
Assoc. Prof. Tomáš Policar

Dean of Faculty of Fisheries and Protection of Waters:
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Name:
Swapnil Gorakh Waghmare

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Ageing is associated with substantial changes in the physiology of the cells (Frenk and Houseley, 2018). These changes gradually decrease the cellular functional abilities, which makes cells or tissue more susceptible to environmental phenomena, reduces the probability of survival and ultimately leads to death (Shay and Wright, 2007). The oocyte, as a single cell, undergoes ageing with increasing time following ovulation. The time span between ovulation and fertilization, known as postovulatory oocyte ageing, leads to changes in maternally incorporated egg components and thereby a drop in egg quality. Egg quality is an inevitable factor in fish farming in regard to aquaculture production success and offspring health (Kjørsvik et al., 1990; Bobe and Labbe, 2010; Migaud et al., 2013; Valdebenito et al., 2013). Dynamic processes associated with oogenesis have been extensively studied in fish (*e.g.*, Wallace and Selman, 1990; Lyman-Gingerich and Pelegri, 2007; Kagawa, 2013). Oogenesis includes the development of primordial germ cells (PGCs), synthesis of yolk protein precursors and their processing within the developing oocyte, deposition of vitamins in eggs, structure and function of egg envelopes and oocyte maturation processes, and maternal investment in oocytes and eggs (reviewed by Lubzens et al., 2010, 2016). In short, the process of oogenesis includes the migration of undifferentiated PGCs to the ovaries and generation of differentiated oogonia, formation of ovarian follicles through engulfing by gonadal somatic cells, mitotic division of diploid oogonia, formation of the primary oocyte through first meiotic division of diploid oogonia, meiotic arrest of the primary oocyte at the end of the prophase in the diplotene stage, accumulation of the informational components and nutritional reserves (vitellogenesis), completion of first meiotic division and formation of the secondary oocyte, and maturation and the ovulation of the secondary oocyte. Regulation of these complex activities associated with oocyte development and maturation involves pituitary gonadotropins and sex steroids (Yaron and Levavi-Sivan, 2011). Molecular mechanisms regulating hormonal cross-talk during oocyte development until fertilizable egg production have been well described (Kumar and Betsy, 2020).

Determinants of fish egg quality have been studied for decades and are still being investigated. Numerous extrinsic and intrinsic factors can influence egg quality in fish (Bobe and Labbe, 2010; Valdebenito et al., 2013; Reading et al., 2018). After releasing oocytes from ovarian follicular cells, the time duration to fertilization has been identified as the most determinant parameter of egg quality (Tarin et al., 2000; Migaud et al., 2013; Valdebenito et al., 2013; Samarin et al., 2015). Oocyte ageing leads to a time-dependent progressive loss in egg quality after ovulation, which makes oocytes less likely to be fertilized and, if fertilized, more likely to produce abnormal embryos (Wilcox et al., 1998; Fissore et al., 2002; Bobe and Labbe, 2010; Samarin et al., 2015). The oocytes remain optimally fertilizable for a period that varies greatly in different fish species and is affected by the storage temperature (Samarin et al., 2015).

The knowledge of egg phenotype and functional changes associated with oocyte ageing has been reviewed in fish (Samarin et al., 2015) and in other vertebrates (Tarin et al., 2000). However, the investigation and understanding of the molecular mechanisms underlying oocyte ageing need more consideration. Oxidative stress is proposed as a possible initiator of the cascade of events that occur during the oocyte ageing process in mice and pigs (Takahashi et al., 2003; Cui et al., 2011). Mitochondrial oxidative phosphorylation is the primary source of energy in the oocyte (Dumollard et al., 2007a,b). This oxidative phosphorylation produces most of the cellular reactive oxygen species (ROS) as a byproduct. Furthermore, it is suggested that other environmental factors, such as exposure to light, lack of antioxidants, and increased oxygen, lead to oxidative stress in postovulatory aged oocytes of rabbits,

rhesus monkeys, and mice (reviewed by Lord and Aitken, 2013). Oxidative stress during postovulatory oocyte ageing deteriorates oocyte fertilizing ability due to its effect on several cellular fundamental components, such as DNA and proteins, in mice (Lord and Aitken, 2013), pigs and humans (Homer, 2021; Wang et al., 2021). Nevertheless, the biochemical indicators of lipid peroxidation, protein oxidation and antioxidant enzyme activities do not show significant changes during oocyte ageing in the common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) (Samarin et al., 2019a,b). Moreover, transcriptomic analyses revealed no differences in the mRNA abundance of the selected genes related to oxidative stress in common carp (Samarin et al., 2019a) and African catfish (*Clarias gariepinus*) (Samarin et al., 2018). These authors concluded that oxidative stress is unlikely to be the initiator of the oocyte ageing process but most likely appears in the later stages as a consequence of postovulatory ageing (Samarin, 2018).

Epigenetic mechanisms are key regulators of gene transcription, with particular significance in responses to altered environmental signals (Jaenisch and Bird, 2003). Postovulatory ageing as an environmental factor may lead to altered epigenetic modification in aged oocytes. In recent decades, considerable evidence has revealed that ageing, which is deeply controlled by genetics, may also be influenced by (and at the same time influence) epigenetics (Bellizzi et al., 2019). Epigenetics regulate gene expression not by changing the DNA sequence but by changing the conformation of the chromosomes through alterations in DNA methylation patterns, posttranslational modifications of histones and noncoding RNAs (Fig. 1) (Lopez-Otin et al., 2013; Ge et al., 2015). Gametogenesis and early embryonic development involve extensive reprogramming by these epigenetic regulators (Labbé et al., 2017). The establishment of proper epigenetic marks contributes to the postfertilization success and the development of healthy offspring (Ge et al., 2015; Labbé et al., 2017).

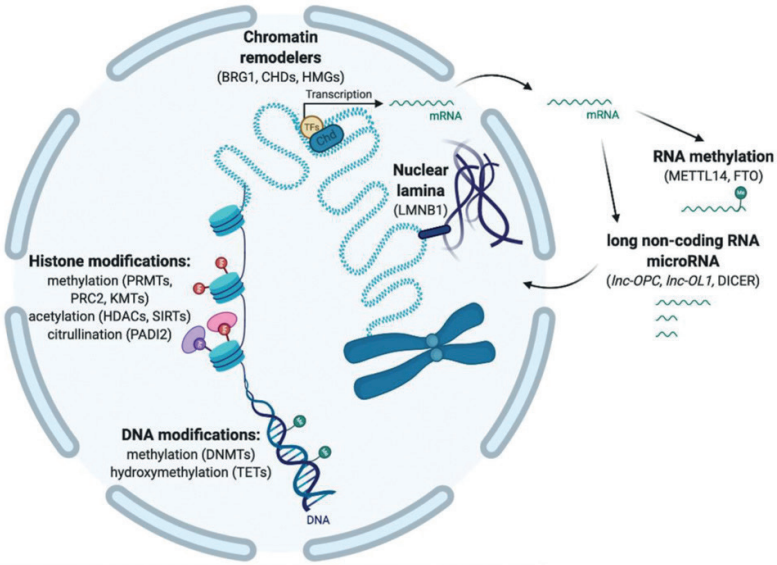

Figure 1. *Mechanisms of epigenetic regulation (obtained from Pruvost and Moyon, 2021).*

1. DNA methylation

Previous reports on oocyte ageing have indicated alterations in DNA methylation patterns in mice and bovines (Imamura et al., 2005; Liang et al., 2008; Heinzmann et al., 2015). DNA methylation refers to the covalent binding of a methyl group to a DNA base, mostly cytosine followed by guanine (CpG islands). Methylation mainly occurs in the promoter regions of genes and repetitive sequences in DNA. These marks are important for gene regulation, proper chromatin condensation and genomic stability (MacKay et al., 2007). The appropriate establishment of DNA methylation patterns in the oocyte is necessary for proper development and survival of the developing embryo (Lopes et al., 2009). DNA methylation patterns are erased and re-established during embryo development (reviewed by Morgan et al., 2005), while genome-wide DNA methylation has not yet been studied in association with oocyte ageing. The reprogramming of DNA methylation initially starts during gametogenesis; mature gametes are heavily methylated, and after fertilization, demethylation starts to occur (Santos et al., 2002). DNA methylation dynamics during embryo development in model fish species such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) have been reported (Fang et al., 2013; Wang and Bhandari, 2019). A previous study indicated that imprinted genes undergo demethylation in mouse oocytes during postovulatory ageing (Liang et al., 2008). These authors investigated the DNA methylation status of the maternally imprinted genes *snrpn* and *peg1/mest* and observed a significant demethylation of differentially methylated regions of *snrpn* during the process of postovulatory ageing. However, the methylation pattern for *peg1/mest* was shown to be not affected. Imamura et al. (2005) reported dynamic changes in DNA methylation of *peg1/mest* during mouse postovulatory oocyte ageing *in vitro.*

DNA methylation patterns are written and erased by DNA methyl transferases (DNMTs) and dioxygenases (TET family proteins) (Labbé et al., 2017). As a result of oocyte ageing, the expression of DNA methyltransferases (*dnmts*) is affected. Heinzmann et al. (2015) suggested that *dnmt3L* is a key molecule that is influenced by postovulatory ageing in bovine oocytes. Transcript levels for *dnmt3a* were found to be significantly reduced in bovine embryos arising from postovulatory aged oocytes (Heinzmann et al., 2015). The authors then indicated that the altered *dnmt3L* gene results in altered DNA methylation and imprinting. In mice, the altered expression of *dnmt3L* and hypermethylation affects offspring viability (Liang et al., 2011). The expression of *dnmt*s has been reported to be decreased in both maternally aged and postovulatory aged oocytes in mice (Hamatani et al., 2004; Pan et al., 2008). Altogether, postovulatory oocyte ageing causes a decline in reproductive outcomes but does not evidently disrupt the acquisition of methylated imprints in oocytes from viable offspring (Liang et al., 2012). For future research works, it will be interesting to determine whether global DNA methylation in oocytes and embryos is affected by postovulatory ageing.

2. Histone modifications

Posttranslational histone modifications can modify chromatin activity and therefore have important roles in regulating gene expression and various cellular functions, which in turn control the development of the developing embryo (Grunstein, 1997; Peterson and Laniel, 2004; Cedar and Bergman, 2009). Histones are the basic nuclear proteins involved in the packaging of DNA into histone octamers, nucleosomes, and chromatin fibres to form the chromosome structure (Fig. 2). Positively charged histone proteins attract electrostatic interactions with the negatively charged DNA and facilitate chromatin condensation. The chromatin structure is comprised of four core histones (H2A, H2B, H3, and H4), the linker histone H1 and DNA. Histone octamers are formed by combining the two sets of individual

core histone proteins. The 147 base pairs of the DNA strand wind around the octamers to form the nucleosome structure. The nucleosomes are further connected with the linker histones (H1) to form the chromatin fibre. The structured globular domain of the core histones facilitates histone–histone interactions within the octamer, along with interactions with the DNA phosphodiester backbones (Cao and Dang, 2018). An amorphous 20–35 amino acid N-terminal tail protrudes from the surface of the nucleosome (Peterson and Laniel, 2004).

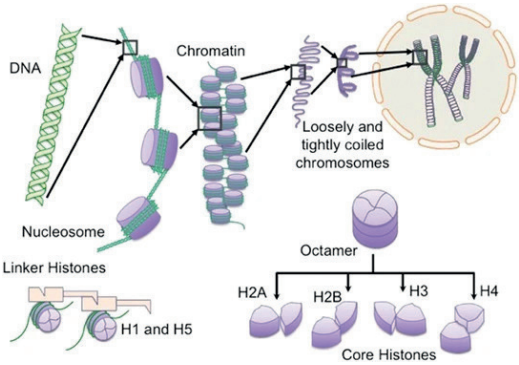

Figure 2. *Intracellular structure and function of histone proteins (obtained from Silk et al., 2017).*

The N-terminal amino acid tails of the histone proteins undergo various posttranslational modifications (PTMs) (reviewed by Rothbart and Strahl, 2014). The N-terminal amino acid chain of histone proteins is subjected to various posttranslational modifications, such as acetylation, phosphorylation, methylation, ADP-ribosylation, ubiquitination, sumoylation, biotinylation and proline isomerization (Fig. 3). These PTMs on histone proteins play a crucial role in controlling DNA functions (Rothbart and Strahl, 2014). Not only gene expression but also other processes, such as DNA repair, replication and recombination, can also be regulated by these modifications (Bannister and Kouzarides, 2011). Histone PTMs act via two major mechanisms: one involves the alteration of the overall chromatin structure, and the other regulates the binding of effector molecules through histone posttranslational modifications (Bannister and Kouzarides, 2011; Allis and Jenuwein, 2016). Acetylation and methylation are the most abundant modifications on histones (Li, 2002; Shiota and Yanagimachi, 2002). Different histone modifications occur dynamically during oocyte maturation (Gu et al., 2010) and initial embryo development (Sarmento et al., 2004).

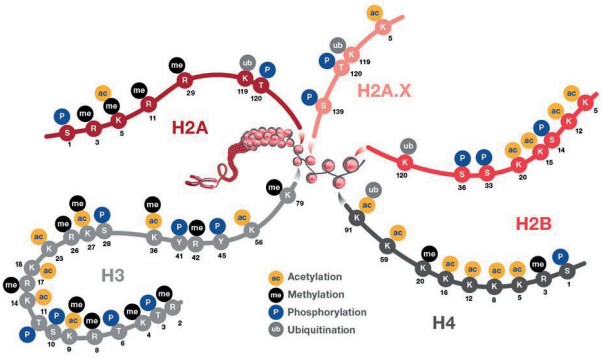

Figure 3. *Important sites of histone posttranslational modifications affecting epigenetics (obtained from Thermo Fisher Scientific, 2021).*

2.1. Histone acetylation

Acetylation was the first kind of histone PTM discovered (Allfrey et al., 1964). Histone acetylation is the most widely studied modification, as it appears more frequently than any other modification (Gavazzo et al., 1997; Turner, 2000; Bannister and Kouzarides, 2011). The specific lysine residues on the N-terminal amino acid chain receive acetylation modifications (Strahl and Allis, 2000). Two lysines on histone H3 (K9, K14) and four lysines on histone H4 (K5, K8, K12, K16) are the predominant epigenetic regulator sites (Gu et al., 2010). It is generally believed that acetylation of histones is characteristic of active transcription of the associated genes, whereas deacetylation of histones relates to gene silencing (Delage and Dashwood, 2008).

Posttranslational addition of the acetyl group to the N-terminal histone tail has diverse roles at the molecular level. The first important role is during the deposition of the newly synthesized histone proteins in the chromatin structure. Another important function of histone acetylation is to promote transcription by favouring a more open chromatin conformation that permits binding of the transcriptional machinery (reviewed by Cao and Dang, 2018). This is done by neutralizing the positive charge on histone tail lysines, which results in the opening of the chromatin structure and allows the binding of transcription factors (Vettese-Dadey et al., 1996). In addition, acetylation on histones serves as a binding site for proteins involved in gene activation (Kurdistani and Grunstein, 2003). Previous studies have demonstrated that with the progression of oocyte ageing, either *in vivo* or *in vitro*, more acetylation modifications on histones are observed (Liang et al., 2012). Specific histone acetylations have been reported to be altered during oocyte ageing (Liang et al., 2012; Ge et al., 2015). Ageing of mouse oocytes results in increased acetylation of H3K9 (Xing et al., 2021), H3K14, H4K8 (Huang et al., 2007), and H4K12 (Huang et al., 2007; Liu et al., 2009). Similarly, acetylation of H4K12 increases significantly during *in vitro* oocyte ageing in pigs (Cui et al., 2011). Artificially delayed oocyte ageing in a mouse model results in hypoacetylation at lysine residues (Huang et al., 2007). In mice, it was also demonstrated that the artificial acceleration of oocyte ageing progress correlates with hyperacetylation at lysine residues (Yoshida et al., 1990; Huang et al., 2007). This evidence indicates that altered histone acetylation might contribute to the progression of postovulatory oocyte ageing.

Matured oocytes are transcriptionally inactive until fertilization (Gilbert, 2000). The zygotic clock is a molecular clock that initiates cascades of biochemical processes that occur postfertilization or after egg activation (Schulz and Harrison, 2019). Embryonic transcription begins at the time of zygotic genome activation at a specific time, depending on the organism (reviewed by Jukam et al., 2017). Posttranslational histone modifications are among the mechanisms involved in zygotic genome activation at the correct time after fertilization (Sato et al., 2019). An increase in histone acetylation is expected during the maternal to zygotic transition and has been reported to be associated with the activation of genes (Fig. 4) (Lee et al., 2014b; Sato et al., 2019). Therefore, it is postulated that postovulatory oocyte ageing may be one of the factors that spontaneously releases the zygotic clock, thereby triggering molecular pathways and leading to the foremost consequences (Demond et al., 2017).

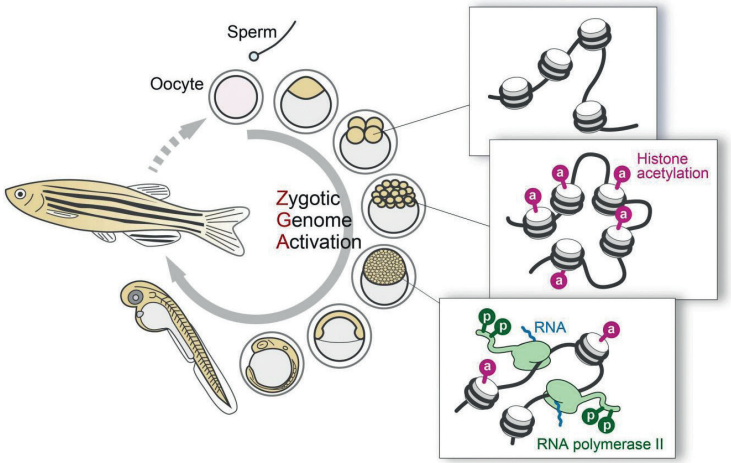

Figure 4. *Histone acetylation precedes active transcription during zygotic genome activation (obtained from Sato et al., 2019).*

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are among the multiple enzymatic systems assuring the generation and maintenance of epigenetic patterns. The balance between acetylation and deacetylation modifications is achieved by the activity of these enzymes. The most reliable functional characteristic of HATs is that they are transcriptional coactivators (Berger, 2007). Based on the subcellular location, HATs are classified into two groups, HAT A and HAT B (Cao and Dang, 2018). The HATs in type A are located in the nucleus and are further classified into three families, the GNAT (Gcn5-related N-acetyltransferase) superfamily, MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60), and p300/CBP (Sterner and Berger, 2000). The HATs in the cytoplasm are categorized as type B and are responsible for the acetylation of newly synthesized histones during the process of chromatin assembly (Parthun, 2007). HAT1 is an evolutionarily conserved enzyme categorized under the HAT type B (Parthun, 2007). Oocyte ageing in other vertebrates is reported to induce alterations in HATs and HDACs. Cui et al. (2011) reported an increased level of *hat1* transcripts in aged porcine oocytes compared to fresh oocytes. A similar surge in *hat1* transcript levels was observed in *in vitro* aged goat oocytes (Zhang et al., 2013).

The HDACs remove the acetyl groups from the N-terminal lysine amino acids and are categorized into four classes, class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 6, 7, 9, 10), class III, also called sirtuins (SIRT1, 2, 3, 4, 5, 6, 7) and class IV (HDAC11), based on their homology to yeast proteins (Zhao et al., 2018). Manosalva and Gonzalez (2010) showed that the expression of *sirt1* decreases in maternally aged mouse oocytes. *sirt1* was also shown to protect mouse oocytes against oxidative stress during maternal ageing (Di Emidio et al., 2014). Furthermore, the genes involved in histone deacetylation, *sirt1*, *sirt2*, and *sirt3,* were found to be downregulated during *in vivo* and *in vitro* oocyte ageing in mice (Zhang et al., 2016; Wang et al., 2017). Specific inhibitors of HDACs can delay oocyte ageing in mice and pigs (Huang et al., 2007; Jeseta et al., 2008; Lee et al., 2014a). The fact that HDACs regulate the life length of different organisms has shed new light on the mechanisms of ageing (Huang et al., 2007). Alterations in the expression of genes involved in the deacetylation of histones might play an important role in extending the longevity of animals (Chang and Min, 2002). Therefore, investigating the possible histone modifications would be a step forward in better understanding the epigenetic mechanisms behind fish oocyte ageing.

2.2. Histone methylation

Methylation is another essential modification that appears on histones and is involved in the regulation of transcription. The lysine and arginine residues on the N-terminal tails are the sites that receive the methylation modifications on histone proteins. Histones can be mono-, di- or trimethylated at lysines and mono- or di-methylated at arginines (Peterson and Laniel, 2004; Bannister and Kouzarides, 2011). These methyl marks are regulated by a group of enzymes, protein arginine methyltransferases (PRMTs) and lysine methyltransferases (KMTs) (Bannister and Kouzarides, 2011; Cao and Dang, 2018). The addition of the methyl group does not alter the overall charge of the histone proteins (Cao and Dang, 2018). Methylation of specific lysine and arginine residues on the histone N-terminal tail has a key role in DNA repair, the cell cycle, stress response, development, differentiation, and ageing (reviewed by Greer and Shi, 2012).

The methylation status of different lysine and arginine residues on histones H3 and H4 has been assessed in mice (Sarmento et al., 2004; Kageyama et al., 2007; Ooga et al., 2008), pigs (Park et al., 2009), sheep (Hou et al., 2008), and humans (Qiao et al., 2010). A study in zebrafish suggests that early embryo developmental instructions may be encoded by specific histone modifications, such as H3K4me3, H3K9me3, and H3K27me3 (Lindeman et al., 2011). Furthermore, the dynamic regulation of H3K4me3 and H3K27me3 has been reported in zebrafish gametes and during early embryonic development (Zhu et al., 2019). Furthermore, histone methylation is reported to be constant during oocyte maturation (Gu et al., 2010), while the methylation status during oocyte ageing is altered (Petri et al., 2020). General histone methylation decreases with maternal oocyte ageing (Ge et al., 2015). A previous study showed a decrease in H3K9 trimethylation in postovulatory aged mouse oocytes (Trapphoff et al., 2016). In contrast, Petri et al. (2020) reported that *in vitro* postovulatory ageing induces an increase in H3K9 tri-methylation in mouse oocytes and the developing two-cell embryos (Petri et al., 2020). Wang et al. (2017) showed that the potent antioxidant quercetin is able to delay oocyte ageing and improve the quality of the resulting embryos. Such a delay is caused by preventing the decrease in *sirt* expression and methylation of histones. In addition, the expression of the histone methylation-related factors *cbx1* and *sirt1* proteins was shown to increase and decrease, respectively, during the maternal ageing process in germinal vesicle stage mouse oocytes (Ge et al., 2015).

3. Non-coding RNAs

Noncoding RNAs have been classified as epigenetic modulators in several studies because they affect the protein levels of target mRNAs without affecting their DNA sequence (reviewed by Morales et al., 2017). Some noncoding RNAs, such as microRNAs (miRNAs), have been reported to be involved in the differentiation and maturation of various cell types (Jima et al., 2010; Takacs and Giraldez, 2010). In addition, miRNAs have a role in regulating embryogenesis (Giraldez et al., 2005; Schier and Giraldez, 2006). Noncoding RNAs are suggested to function in general ageing (Kato and Slack, 2013). Transcriptomic profiling during ova ageing in rainbow trout *Oncorhynchus mykiss* suggested the dysregulation of miRNAs and their target genes involved in cell death, signal transduction, stress response, DNA damage and transcription regulation (Ma et al., 2015). Furthermore, a recent advanced database, FishmiRNA (Desvignes et al., 2022), could be utilized to explore miRNA profiling in fish gametes.

The examination of oocyte ageing consequences is practically difficult to achieve in higher vertebrates. In addition, it is ethically not justifiable in many cases. Therefore, the necessity of finding suitable animal models to investigate the phenomena is apparent. The high number

of oocytes produced and the easy manipulation of oocytes *in vitro,* as well as the intrinsic nature of external fertilization in fish, make the study of oocyte ageing in fish effective and more convenient compared with other model organisms. Zebrafish may be one of the most suitable model organisms to investigate oocyte ageing. The well-known genome and functional similarities of genes with humans make zebrafish a model of interest to study oocyte ageing at the molecular level. Furthermore, the easy manipulation of the organism during the experiment compared to other vertebrates make zebrafish appropriate for these studies. Additionally, the virtually transparent and rapid development of embryos enable researchers to better investigate the effect of oocyte ageing on initial embryo development at the phenotypic level. Common carp and grass carp are among the major aquaculture species, and investigating histone modifications could lead to the discovery of epigenetic/biological markers for egg quality associated with oocyte ageing, which could possibly apply to other species as well.

The present study investigated the egg phenotypic and functional properties (fertilization, eyeing, hatching, embryo mortality, larval malformation, ploidy anomaly percentages) during oocyte ageing in zebrafish (*Danio rerio*) (Chapter 2), common carp (*Cyprinus carpio*) (Chapter 3) and grass carp (*Ctenopharyngodon idella*) (Chapter 4). The epigenetic mechanisms responsible for the effects of postovulatory oocyte ageing were investigated, with a focus primarily on histone acetylation. After analysis of the global histone modification level, blotting methods (Western blotting) were used to detect differences in expression to identify candidate proteins for a more detailed investigation of acetylation-specific differences in histone modification. The probable changes in the acetylation patterns of selected lysine residues on histones H3 (K9, K14) and H4 (K5, K8, K12, K16) during the ageing of common carp (Chapter 3) and grass carp (Chapter 4) oocytes were examined. To add more information to the context of histone acetylation and oocyte ageing, histone acetyltransferase enzyme activity was assessed during *in vivo* and *in vitro* oocyte ageing in common carp and during *in vitro* oocyte ageing in grass carp. To date, the current study is the first to elaborate histone modifications as a crucial epigenetic regulator during fish oocyte ageing. Knowledge of the molecular mechanisms that contribute to critical phenotypic and functional changes associated with oocyte ageing will benefit both basic and applied research in aquaculture as well as assisted reproduction technology in other vertebrates.

4. Thesis aim and specific objectives

The current study was devoted to investigating the possible alteration in histone modifications during fish postovulatory oocyte ageing. Investigations were performed on different aged oocytes, and the embryos originated from those oocytes.

Specific objectives

1. Examining the egg phenotype and functional changes during oocyte ageing in zebrafish, common carp, and grass carp.
2. Examining global histone modifications during oocyte ageing in common carp.
3. Examining the specific histone acetylation patterns (AcH3K9, AcH3K14, AcH4K5, AcH4K8, AcH4K12, AcH4K16) during oocyte ageing in common carp and grass carp.
4. Examining the activity of histone acetyltransferase during oocyte ageing in common carp and grass carp.

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CHAPTER 2

OOCYTE AGEING IN ZEBRAFISH *DANIO RERIO* (HAMILTON, 1822) AND ITS CONSEQUENCE ON THE VIABILITY AND PLOIDY ANOMALIES IN THE PROGENY

Waghmare, S.G., Samarin, A.M., Franěk, R., Pšenička, M., Policar, T., Linhart, O., Samarin, A.M., 2021. Oocyte ageing in zebrafish *Danio rerio* (Hamilton, 1822) and its consequence on the viability and ploidy anomalies in the progeny. Animals 11, 912.

My share on this work was 40%.

animals

MDPI

Article

Oocyte Ageing in Zebrafish *Danio rerio* (Hamilton, 1822) and Its Consequence on the Viability and Ploidy Anomalies in the Progeny

Swapnil Gorakh Waghmare, Azadeh Mohagheghi Samarin, Roman Franěk, Martin Pšenička, Tomáš Policar, Otomar Linhart and Azin Mohagheghi Samarin *

Research Institute of Fish Culture and Hydrobiology, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Zátiší 728/II, 389 25 Vodňany, Czech Republic; swaghmare@frov.jcu.cz (S.G.W.); amohagheghi@frov.jcu.cz (A.M.S.); franek@frov.jcu.cz (R.F.); psenicka@frov.jcu.cz (M.P.); policar@frov.jcu.cz (T.P.); linhart@frov.jcu.cz (O.L.)

* Correspondence: mohagheghi@frov.jcu.cz

Simple Summary: The maintenance and manipulation of AB strain zebrafish oocytes at 26 °C was found to be possible for 2 h without incurring a marked reduction in fertilization potential. However, the post-ovulatory ageing of oocytes for 6 h resulted in an almost complete loss of egg viability. All larvae derived from the 4- and 6-h aged oocytes were characterized by physical abnormalities. Ageing oocytes for 4 h resulted in the incidence of ploidy anomalies having a four-fold increase. These results make a valuable contribution with respect to the control of experimental reproduction in zebrafish, which is currently accepted as an excellent model animal.

Abstract: Fish egg quality can be markedly influenced by the oocyte age after ovulation. In this study, we examined the duration of oocyte ageing in the zebrafish (*Danio rerio*) and whether prolonged ageing is associated with the incidence of ploidy anomalies in the resulting embryos. Oocytes were incubated in vitro for 6 h post-stripping (HPS) at 26 °C and fertilized at 2-h intervals. Meanwhile, for eggs fertilized immediately after stripping, the fertilization, embryo survival, and hatching rates started at ~80%; these rates decreased to 39%, 24%, and 16%, respectively, for oocytes that had been stored for 4 h ($p < 0.05$), and there was an almost complete loss of egg viability at 6 HPS. Furthermore, almost 90% of the embryos derived from 6-h aged oocytes died prior to hatching, and all larvae originating from 4- and 6-h aged oocytes showed malformations. The proportion of ploidy abnormal embryos was significantly greater at 4 HPS (18.5%) than at either 0 or 2 HPS (4.7% and 8.8%, respectively). The results revealed that zebrafish oocytes retained their fertilization potential for up to 2 h after stripping at 26 °C and indicated the contribution of post-ovulatory oocyte ageing in the occurrence of ploidy anomalies in the resulting embryos.

Keywords: egg storage; embryo development; ploidy anomalies; progeny abnormality; zebrafish AB strain

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1. Introduction

Oocytes, the gametes of female organisms, are the final products of oogenesis, during which all essential components, such as maternal mRNAs, proteins, lipids, carbohydrates, vitamins, and hormones, that support initial embryonic development are incorporated [1]. These maternally incorporated endogenous factors are important for ensuring the production of good-quality eggs, and they subsequently contribute to successful fertilization and successful embryo development [2,3]. Oocytes undergo an ageing process with a prolongation in the time span between ovulation and fertilization that is known as post-ovulatory oocyte ageing, during which there is an accumulation of changes in the maternally derived

egg components and a concomitant reduction in egg quality. Oocyte ageing appears to be a complex process involving multiple pathways. Some of the cellular and molecular changes associated with this ageing process have been investigated in fish [4–6], as well as in other vertebrates [7,8]. However, the precise pathways underlying oocyte ageing have yet to be sufficiently characterized.

Limited fertilization capacity [9–11] and disturbed embryonic development [12,13] are among the major phenotypical and functional consequences of oocyte ageing. Prolonged or retracted development during embryogenesis is frequently observed in embryos arising from more aged oocytes. Moreover, although oocytes that undergo relatively shorter periods of post-ovulatory ageing can still produce eyed and hatched embryos, these can be prone to post-hatching abnormalities. For example, fish larvae that develop from aged oocytes are often characterized by corporal malformations [9,12,14], and ploidy anomalies in progeny have been reported among the detrimental outcomes of oocyte ageing, with an increased occurrence of ploidy anomalies being observed in trout (*Oncorhynchus mykiss*) [14], tench (*Tinca tinca*) [15], Japanese eel (*Anguilla japonica*) [16], northern pike (*Esox lucius*) [17], and yellowtail tetra (*Astyanax altiparanae*) [13]. However, the consequence of oocyte ageing on ploidy abnormalities has not yet been studied in zebrafish despite it being widely used as a model organism. Oocyte ageing may also have long-lasting repercussions on progeny, leading to a heightened susceptibility to certain disorders later in life [18].

Zebrafish are valued for their ornamental characteristics, but they mainly serve as an excellent model animal basic biology research [19–21]. Given their small size, the housing and care of zebrafish are relatively simple and inexpensive. Additionally, unlike many other fish species, zebrafish mature rapidly within two to four months, and they can also reproduce throughout the year. The short life cycle of this tiny fish thus makes it eminently suitable for multi-generational studies [22]. Moreover, zebrafish eggs, embryos, and larvae are transparent, which is conducive for experimental observations. In comparison with other model organisms, zygotic genome activation is delayed in zebrafish, thereby enabling research, whereas embryonic development is still controlled by maternal components [23]. Furthermore, the feasibility of zebrafish egg storage enables the provision of synchronized female and male gametes in artificial reproduction schedules, which also has advantages with respect to the control of experimental reproduction and the manipulation of oocytes in associated studies. Capitalizing on the aforementioned advantages, we used zebrafish in the present study to investigate the effect of post-ovulatory oocyte ageing on egg fertilization potential, embryo development, larval malformations, and the incidence of ploidy anomalies in progeny.

2. Materials and Methods

The effect of post-postovulatory oocyte ageing on the viability and ploidy anomalies in the zebrafish (AB strain) progeny was studied. Following ovulation, oocytes obtained from 7 females were stored separately in vitro for 6 h and artificially inseminated with 2 h-intervals.

2.1. Broodfish

Generations from zebrafish (AB strain) that were initially obtained from the European Zebrafish Resource Center (Karlsruhe, Germany) were used as the broodstock. The fish were kept in the rearing system (ZebTec Active Blue). The fish were cultured under controlled conditions (14-h light:10-h dark photoperiod; temperature: 28 °C; feeding: TetraMin flakes twice daily and Artemia nauplii once daily). The experimental fish were aged approximately 12 months with an average weight of 0.57 g (males) and 0.81 g females). During the afternoon of the day prior to commencing experiments, 10 pairs of broodfish were separately placed (one male and one female) into 1-L spawning chambers, within which the males and females were initially separated using a barrier. At light onset on the following day, the barriers were removed, and the fish were thereafter inspected for ovulation. Upon observation of a few eggs at the bottom of a chamber, the females (7)

were assumed to have ovulated. The experimental fish were immediately anaesthetized using 0.05% tricaine methanesulfonate (methyl-aminobenzoate, MS222) for the purpose of gamete collection [24].

2.2. In Vitro Oocyte Ageing and Sampling

Ovulated oocytes were collected from each female by hand stripping and separately placed into sterile Nunc glass-bottomed dishes (40 mm in diameter) (Thermo Fisher Scientific, Rochester, NY, USA), without the addition of any artificial media. A small piece of moistened tissue was placed adjacent to the eggs to maintain a sufficient level of humidity. Having replaced the lids, the dishes were stored in a laboratory incubator (Q-cell, 140/40 Basic; Wilkowice, Poland) at 26 °C for 6 h. Stored oocytes were fertilized at 0 (immediately after stripping), 2, 4, and 6 h post-stripping (HPS).

2.3. Artificial Insemination

Prior to artificial fertilization, the stripped milt from each male was examined for sperm motility under a Nikon SMZ745T stereomicroscope (Nikon, Japan) according to Fauvel et al. [25]. Given that zebrafish males generally produce small quantities of milt (typically not exceeding 2 µL) and to provide a uniform fertilization potential for all egg batches, sperm was collected from five males and pooled to give a composite sample for the purposes of artificial fertilization at each time point. The pooled milt was added to a tube containing 50 µL of an immobilizing solution (Kurokura 180 solution) [26]. For the artificial fertilization at each HPS, batches of ~100 oocytes were placed in Petri dishes, to which 10 µL of the pooled sperm followed by 0.2 mL of water were added. Having been shaken for 1 min, egg batches were washed by immersing in 3 mL of water. Preliminary evaluations enabled us to confirm that the oocyte:sperm:water ratio used in the present study was sufficient to ensure the fertilization of all eggs. The eggs thus inseminated were thereafter maintained in Petri dishes (9 cm in diameter) at 28 °C in a laboratory incubator.

2.4. Examination of Egg Developmental Success and Larval Quality

As egg quality indices, we examined the rates of fertilization, embryo survival, hatching, embryo mortality, and larval malformation for eggs originating from oocytes of different ages. The calculations were made as below:

(1) Fertilization%: (number of eggs showing cleavage/total number of inseminated eggs) × 100.
(2) Embryo survival%: (number of 24-h live embryos/total number of inseminated eggs) × 100.
(3) Hatching%: (number of hatched larvae/total number of inseminated eggs) × 100.
(4) Egg mortality%: (number of dead embryos/total number of 24-h survived embryos) × 100.
(5) Larval malformation%: (number of morphologically abnormal larvae/total number of hatched larvae) × 100.

Fertilization rates were assessed for each egg batch 90 min after egg insemination, based on the occurrence of cell divisions observed under a Nikon SMZ745T stereomicroscope. Egg batches were re-examined at 24 h after insemination to evaluate embryo survival. The embryo mortality was measured between 24 h after the insemination and the hatching. After hatching (48–72 h after insemination), larvae were observed under a stereomicroscope in order to determine the occurrence of any morphological abnormalities (e.g., shortened body, deformed tail, spinal cord torsion, deformed yolk sac, and eye deformations) [27], thereby facilitating an estimate of the extent of larval malformation.

2.5. Examination of Ploidy Levels in Progeny

The potentially adverse effects of post-ovulatory oocyte ageing on the ploidy level of progeny emerging from eggs developed from oocytes of different ages were assessed on the first day post-fertilization and again immediately after hatching, based on flow cytometric

measurements of the relative DNA content of nuclei. Detailed information relating to the number of analyzed embryos and larvae derived from oocytes at each HPS is presented in Table 1. The experimental embryos and larvae were processed using a CyStain UV Precise T kit for nuclei staining (Sysmex Partec GmbH, Münster, Germany) according to the manufacturer's protocol, with the following modifications. Single embryo/larva were homogenized in 200 µL of the extraction buffer. Samples were then filtered through 30-µm mesh filters (Partec Cell Trics disposable filter units; Partec). Subsequently, 1 mL of the staining buffer was added to the nuclei suspension and analyzed by flow cytometry at a flow rate of 0.4 µL s^{-1}. The relative DNA content was determined using a CyFlow Ploidy Analyser (Sysmex Partec GmbH) against samples from the diploid control group (eggs fertilized at 0 HPS).

Table 1. Effect of post-ovulatory oocyte ageing on ploidy anomalies in the embryos (1-day old) and larvae (immediately post-hatching) of zebrafish.

1 Day Post-Fertilization		
HPS	**No. of Analyzed Embryos**	**Percentage Ploidy Anomalies**
0	64	4.7 (100% Tr)
2	34	8.8 (33% H, 33% Te, 33% M)
4	27	18.5 (40% H, 40% Tr, 20% M)
Larvae Immediately Post-Hatching		
HPS	**No. of Analyzed Larvae**	**Percentage Ploidy Anomalies**
0	40	0
2	65	0
4	20	0

HPS: hours post-stripping; H: haploid; Tr: triploid; Te: tetraploid; M: mosaic.

2.6. Statistical Analysis

Data analysis was performed using the R (Version 4.0.3) statistical program. The normality of the data was assessed using histogram. Following ANOVA, Duncan's multiple range test was used to analyze the data, and differences were considered significant at the $p < 0.05$ level.

3. Results

3.1. Egg Quality Indices of Ageing Oocytes

The highest fertilization, embryo survival, and hatching rates (~80%) were observed for those eggs fertilized immediately after stripping (Figure 1). Though not significantly different, we detected a reduction in the rate of fertilization to 64% for the eggs derived from 2-h aged oocytes. Thereafter, the fertilization rates significantly decreased and dropped to only 3% at 6 HPS. The percentages of embryo survival were, however, significantly impaired, decreasing to 51%, 24%, and 2% at 2, 4, and 6 HPSs, respectively. The hatching rates followed the same trend of significant decrease through oocyte ageing, and only less than 1% of the eggs hatched at 6 HPS. Moreover, we observed significant increases in the rates of embryo mortality and larval malformation after 4 HPS, with 73% and 90% of the fertilized eggs originating from the oocytes aged for 4 and 6 HPS, respectively, dying prior to hatching (Figure 2). Meanwhile, only 5% of the control group (0 HPS) larvae showed any evidence of malformation; all the larvae originating from 4- and 6-h aged oocytes were observed to be malformed, mostly suffering from the skeletal abnormalities.

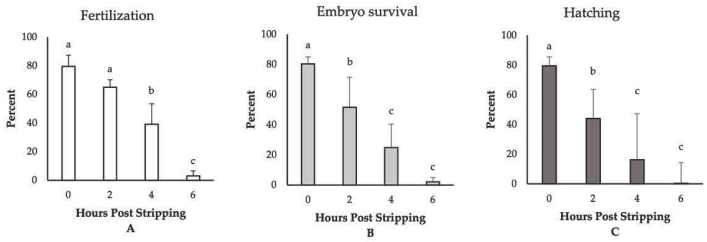

Figure 1. The effect of post-stripping oocyte ageing on the rates of (**A**) fertilization, (**B**) embryo survival, and (**C**) hatching in zebrafish (mean ± SD). Mean values indicated by the same letter (a, b, c) do not differ significantly.

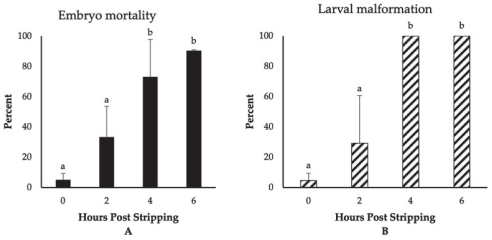

Figure 2. The effect of post stripping oocyte ageing on the rates of (**A**) embryo mortality and (**B**) larval malformation in zebrafish (mean ± SD). Mean values indicated by the same letter (a, b) do not differ significantly.

3.2. Ploidy Anomalies of Ageing Oocytes

One-day-old embryos derived from oocytes aged for 0 and 2 h prior to insemination were characterized by 4.7% and 8.8% ploidy anomalies, respectively (Table 1). Ageing oocytes for 4 h was observed to result in a continuous increase in the percentage of anomalies inone1-day-old embryos, with a four-fold increase (18.5%) in the incidence of ploidy anomalies being detected, as assessed by flow cytometry. A majority of these ploidy anomalies were manifested as triploids (40%) and haploids (40%), whereas the remaining 20% of anomalies were mosaics (n/3n).

In contrast, however, post-ovulatory oocyte ageing appeared to have no appreciable influence on the ploidy status of larvae, with the examination of the larvae (freshly hatched) obtained from seven females at 0, 2, and 4 HPS revealing that all were normal diploids. Due to the very limited number of alive embryos and larvae at 6 HPS, the ploidy levels were not possible to be examined at this time point.

4. Discussion

In fish, an inability to achieve optimal timing for fertilization might occur due to a delay in egg spawning or stripping. This, in turn, would result in oocyte ageing, which has been identified as the main factor that impedes successful fertilization and normal embryonic and larval development. The optimal time for egg fertilization following ovulation is typically species-specific and dependent on individual female and storage temperature [28]. In the current study, we detected no significant differences in the fertilization potential of zebrafish (AB strain) oocytes aged in vitro at 26 °C for up to 2 h after stripping, although we did note a decreasing trend during this period. A number of previous studies have examined the effects of storage conditions on oocyte viability. Sakai et al. [29], for example, assessed the effects of storing wild strain zebrafish oocytes at 23 °C in Hanks' buffered

saline supplemented with 0.5% bovine serum albumin (BSA); they found that the stored oocytes retained a fertilization capacity of up to 85% within 1 h, and after 2 h of storage, the rate of fertilization rate did not significantly differ from that of eggs stored for 1 h. Similarly, Cardona-Costa et al. [30] used a medium containing Hanks' saline supplemented with 1.5 g of BSA and 0.1 g of sodium chloride for the storage of golden zebrafish oocytes at 8 °C; based on a monitoring of fertilization capacity and embryonic development, they reported rapid oocyte ageing after less than 1 h. The authors accordingly recommend that fertilization should be conducted as soon as possible after collecting eggs from females and noted that the strain of experimental zebrafish should be taken into consideration when seeking to determine the optimal period for oocyte fertilization.

There is currently little available information regarding the cellular and molecular processes associated with the lower fertilization rates observed for aged oocytes compared with fresh oocytes. It has, however, been indicated that during the fertilization of aged oocytes, there is an impairment of cytoplasmic Ca^{2+} homeostasis, which is followed by abnormal oscillations in Ca^{2+} that are characterized by a higher frequency but a lower amplitude than those of fresh oocytes [31–33]. Similar oscillations have also been reported as a probable cause of the apoptosis pathway associated with abnormal fertilization [34]. Further factors that could potentially account for the reduced fertilization rate of aged oocytes are an increase in oxidative stress and elevated levels of lipid peroxidation in the oocyte plasma membrane [32]. In response to an increase in lipid peroxidation, there is a reduction in membrane fluidity, which can affect the fusion between spermatozoa and the oolemma, thereby reducing the rate of fertilization [35]. However, although these findings provide a superficial insight into the factors contributing to the poor fertilization capacity of aged oocytes, the underlying mechanisms are likely to be more complex and thus warrant more in-depth studies in the future.

The developmental competence of fish embryos is critically dependent on the integrity of oocytes, and our examination of the effects of zebrafish oocyte ageing on embryo survival and hatching rates in the present study revealed that the corresponding rates were significantly lower in the eggs derived from oocytes aged for 2, 4, and 6 h than those derived from fertilized fresh oocytes. These observations accordingly indicated the pivotal importance of oocyte ageing in the deterioration of egg and embryo quality. In this regard, we observed that oocytes subjected to a 6-h post-ovulatory ageing were characterized by defective cleavages. Indeed, only a few embryos (2%) progressed to the gastrula stage, and further development was rarely observed. In contrast to the findings of the present study, Cardona-Costa et al. [30] found that after only 1 h of oocyte ageing at 8 °C in zebrafish (golden strain), the development of embryos to the mid-blastula stage was reduced to 50% compared with the embryos derived from fresh oocytes. Moreover, these authors reported an embryo survival of only 1.8% at 2 HPS and no embryonic development at 3 and 4 HPS. In the present study, we observed that more than 50% of the embryos derived from 2-h aged oocytes developed to the day after fertilization, which was considerably higher than previously published values and can probably be attributed to differences in storage media, storage temperature, and fish strains. In addition, given that the quality of fish oocytes is likely to be affected by other factors in addition to oocyte ageing, the observed inter-female variability in viability is perhaps not surprising. Previous studies have reported significantly lower hatching rates with an increase in the time elapsed between ovulation and fertilization for oocytes aged both in vivo and in vitro. For example, in species such as rainbow trout [36], Japanese eel [16], northern pike [17], yellowtail tetra [13], and common carp (*Cyprinus carpio*) [37], significant reductions in hatching potential have been observed for eggs with an extension in the time between ovulation and fertilization. In this regard, it has been demonstrated that for a majority of those fish species that have been assessed, a reduction in storage temperature from the natural spawning one results in an extension of the successful egg storage time [12,14,38,39]. Accordingly, in future studies, it would be instructive to examine the effects of storage temperature on the in vitro viability of zebrafish oocytes.

Despite the low fertilization capacity of advanced-aged oocytes, they can still give rise to developed embryos and even hatched larvae. However, such embryos and larvae are typically characterized by prolonged or diminished development during the subsequent stages of embryogenesis and are notably prone to premature mortality and post-hatching aberrations. The extrusion of essential components and proteins of yolk from the oocytes to the ovarian fluid during the ageing period [40] and changes in oocyte biochemistry [10,41] may be among the factors contributing to the poor development of embryos derived from aged oocytes. For example, it is believed that cytoplasmic ageing can substantially hamper normal embryo development [35], as changes in oocyte components at the mRNA and protein levels are detectable with increasing time following ovulation [37,42,43]. In zebrafish, the zygotic genome is activated at the 10th cell cycle division, prior to which embryonic development is controlled by maternal mRNAs and encoded products [2]. Maternal effect genes encode proteins that are determined by the maternal genotype, and their mRNAs influence the phenotypes of offspring [44]. The degradation of many maternal mRNAs that are essential for the proper embryo development is not properly done in aged mouse oocytes [45]. The authors suggested that abnormal patterns of maternal mRNA degradation in aged oocytes can contribute to a reduction in the developmental competence of the resulting embryos, and, consequently, a compromised regulation of maternal genes during post-ovulatory oocyte ageing could affect the developmental potential of the generated embryos.

The results obtained in the present study revealing increased malformation and ploidy anomalies in progeny derived from the more aged oocytes were consistent with the findings of previous studies on other fish species. For example, in species such as the African catfish (*Heterobranchus longifilis*) [46], European catfish (*Silurus glanis*) [9,47], Asian catfish (*Pangasius hypophthalmus*) [48], rainbow trout [14,49], and common carp [37], embryos arising from aged oocytes have been observed to suffer from physical malformations and irregularities. Oocyte ageing in rainbow trout [14], tench [15], Japanese eel [16], northern pike [17], and yellowtail tetra [13] has also been shown to be associated with a heightened occurrence of triploidization. In the present study, we detected no significant differences between the ploidy levels of hatched larvae originating from fresh and aged oocytes, with all hatched larvae being normal diploids. In this regard, although it is generally believed that the viability of triploids is similar to that of diploids in many species of fish [50], the results of the current study indicated that ploidy abnormal embryos derived from aged oocytes are less likely to survive than are diploids. This was previously shown for the European catfish as well [51]. This was apparent from our finding based on flow cytometric analysis, which revealed that the percentage of abnormal ploidy individual decreased as embryos developed to larvae, with no ploidy anomalies being detected among those larvae that hatched. Indeed, all hatched larvae were found to be normal diploids. These observations are consistent with the findings of our study on common carp, which indicated that ploidy abnormal embryos derived from post-ovulatory aged oocytes do not reach the hatching stage (Samarin et al., unpublished data). Thus, it is plausible that it is only triploids that develop as a consequence of post-ovulatory oocyte ageing that are less likely to survive, given that the spontaneous occurrence of triploid larvae can be attributed to several factors [16], among which post-ovulatory oocyte ageing is only one of them. Therefore, the ploidy abnormalities caused by the ageing of oocytes are probably a suitable marker for examining the consequence of oocyte ageing on the arising embryos that are already incompatible with life.

Post-ovulatory aged oocytes exhibit chromosomal abnormalities and misalignment due to meiotic spindle defects that could potentially contribute to higher rates of non-viable embryos post-fertilization [35,52]. These aberrations can increase the risk for chromosome separation defects and aneuploidy [53]. Nomura et al. [16] suggested that the occurrence of cytogenetically atypical progeny in Japanese eels is associated with post-ovulatory oocyte ageing, although this appears to be unrelated to sperm ageing. Using genotypic segregation analysis, they found that triploid larvae are derived from duplication of the maternal alleles

but not paternal ones. These events can be ascribed to the inhibition of second polar body and subsequent fertilization with sperm. Mitochondrial dysfunction and a reduction in ATP levels are important contributors to chromosomal abnormalities and the poor quality of aged oocytes and embryos [54]. Hamatani et al. [43] demonstrated that a reduction in the function of ATP-dependent proteins, such as those comprising microtubules and the cytoskeleton, could result in chromosomal segregation defects and an associated increase in aneuploidy in aged oocytes. Furthermore, Pan et al. [45] demonstrated that the strength of the spindle assembly checkpoint is weakened with an increase in reproductive age in mouse oocytes, with the authors concluding that a higher proportion of errors in microtubule–kinetochore interactions could be considered the underlying basis for an increase in the extent of aneuploidy during maternal ageing. Accordingly, in future studies, it would be of considerable interest to examine molecular pathways associated with the incidence of ploidy anomalies caused by fish oocyte ageing.

5. Conclusions

Post-ovulatory oocyte ageing in zebrafish has a considerable influence on the developmental competence of the resulting eggs. In this study, we found that fertilization rates, embryo survival, and the capacity to reach the hatching stage were markedly reduced with a prolongation of post-ovulatory oocyte ageing. An extension in the time of oocyte ageing of up to 2 h in the maintenance and manipulation of AB strain zebrafish oocytes at 26 °C is, nevertheless, possible without incurring a marked reduction in fertilization potential. In contrast, however, the post-ovulatory ageing of oocytes for 6 h resulted in an almost complete loss of egg viability. The mortality rates of the developing embryos and malformations in the resulting larvae were also found to be adversely affected by post-ovulatory oocyte ageing, with all larvae derived from the 4- and 6-h aged oocytes being characterized by physical abnormalities. We believe that the results obtained in the present study will make a valuable contribution with respect to the control of experimental reproduction in zebrafish, which is currently accepted as an excellent model animal.

Author Contributions: A.M.S. (Azin Mohagheghi Samarin), M.P., T.P., and O.L. conceptualized the experiment. S.G.W., A.M.S. (Azin Mohagheghi Samarin), A.M.S. (Azadeh Mohagheghi Samarin), and R.F. carried out the animal experiment. S.G.W., A.M.S. (Azin Mohagheghi Samarin), and A.M.S. (Azadeh Mohagheghi Samarin) examined ploidy levels. A.M.S. (Azin Mohagheghi Samarin), M.P., T.P., and O.L. supervised the study. S.G.W. and A.M.S. (Azin Mohagheghi Samarin) performed the statistical analysis of the results. S.G.W., A.M.S. (Azadeh Mohagheghi Samarin), and A.M.S. (Azin Mohagheghi Samarin) wrote the first draft of the manuscript, and all the authors revised and commented on the first draft of the manuscript. All authors provided critical feedback and helped to shape the research, analysis and final manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The experimental procedures followed in this study were performed in accordance with the ethical guidelines of the EU-harmonized Animal Welfare Act of the Czech Republic. The unit is licensed (No. 16OZ19179/2016-17214) according to the Czech National Directive (Law against Animal Cruelty, No. 246/1992). All methodological protocols, experimental manipulations, and sampling procedures used in the present study were approved by the expert committee of the Institutional Animal Care and Use Committee of the University of South Bohemia, Czech Republic. The co-authors of this study deal with the manipulation and artificial reproduction of fish, and hold certificates giving them the authorization to work with laboratory animals according to Section 15d Paragraph 3 of Act no. 246/1992 Coll. For the purposes of stripping gametes, fish were anaesthetized with 0.05% tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) to ensure their welfare and minimize any associated stress.

Data Availability Statement: Data from the analysis are available from the corresponding author on request.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 3

HISTONE ACETYLATION DYNAMICS DURING *IN VIVO* AND *IN VITRO* OOCYTE AGING IN COMMON CARP *CYPRINUS CARPIO*

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Histone Acetylation Dynamics during In Vivo and In Vitro Oocyte Aging in Common Carp *Cyprinus carpio*

Swapnil Gorakh Waghmare [1,*], Azin Mohagheghi Samarin [1], Azadeh Mohagheghi Samarin [1], Marianne Danielsen [2,3,4], Hanne Søndergård Møller [2], Tomáš Policar [1], Otomar Linhart [1] and Trine Kastrup Dalsgaard [2,3,4]

1 South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, 389 25 Vodňany, Czech Republic; mohagheghi@frov.jcu.cz (A.M.S.); amohagheghi@frov.jcu.cz (A.M.S.); policar@frov.jcu.cz (T.P.); linhart@frov.jcu.cz (O.L.)
2 Department of Food Science, Aarhus University, Agro Food Park 48, 8200 Aarhus, Denmark; marianne.danielsen@food.au.dk (M.D.); hsm@food.au.dk (H.S.M.); trine.dalsgaard@food.au.dk (T.K.D.)
3 Center of Innovative Food Research, Aarhus University Centre for Innovative Food Research, 8000 Aarhus, Denmark
4 CBIO, Aarhus University Centre for Circular Bioeconomy, 8000 Aarhus, Denmark
* Correspondence: swaghmare@frov.jcu.cz

Abstract: Aging is the most critical factor that influences the quality of post-ovulatory oocytes. Age-related molecular pathways remain poorly understood in fish oocytes. In this study, we examined the effect of oocyte aging on specific histone acetylation in common carp *Cyprinus carpio*. The capacity to progress to the larval stage in oocytes that were aged for 28 h in vivo and in vitro was evaluated. Global histone modifications and specific histone acetylation (H3K9ac, H3K14ac, H4K5ac, H4K8ac, H4K12ac, and H4K16ac) were investigated during oocyte aging. Furthermore, the activity of histone acetyltransferase (HAT) was assessed in fresh and aged oocytes. Global histone modifications did not exhibit significant alterations during 8 h of oocyte aging. Among the selected modifications, H4K12ac increased significantly at 28 h post-stripping (HPS). Although not significantly different, HAT activity exhibited an upward trend during oocyte aging. Results of our current study indicate that aging of common carp oocytes for 12 h results in complete loss of egg viability rates without any consequence in global and specific histone modifications. However, aging oocytes for 28 h led to increased H4K12ac. Thus, histone acetylation modification as a crucial epigenetic mediator may be associated with age-related defects, particularly in oocytes of a more advanced age.

Keywords: *Cyprinus carpio*; egg quality; epigenetics; histone acetyltransferase; histone modifications; post-ovulatory aging

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1. Introduction

High-quality eggs are essential for proper embryo development and good health in the later life of the offspring [1]. Internal components such as maternal proteins or gene transcripts and external factors such as brood stock diet, environmental conditions, husbandry practices, and oocyte aging can influence the quality of oocytes [2]. The age of the oocyte has been recognised as a factor that affects egg quality after ovulation [3,4]. It has been observed that genetic and epigenetic changes within the genome can affect the developmental competence of the eggs, and these changes can be inherited by the offspring [5,6]. Fertilisation occurs within a short time after the release of the metaphase II oocyte from the follicle at ovulation [7]. Delays in fertilisation can result in post-ovulatory oocyte aging. In fish, this type of oocyte aging can occur due to defects in egg spawning or stripping.

Aging is a multifaceted process that is characterised by genetic and epigenetic changes within the genome and that involves various molecular pathways [8]. To date, only a

small number of reports have described the molecular changes that occur during fish oocyte aging [9–14]. Epigenetics, the link between the environment and genes, has been suggested as a likely contributor to the aging phenotype [15,16]. Different epigenetic regulators/marks such as DNA methylation, histone modifications, and non-coding RNAs are associated with the aging process in other organisms. Previous studies on oocyte aging indicated the DNA methylation changes in bovine [17], histone modifications in mice and porcine [18,19], and modified expression of non-coding RNAs in rainbow trout (*Oncorhynchus mykiss*) [12]. The epigenome of an organism is extensively reprogrammed during gametogenesis and early embryo development through these epigenetic regulators/marks. Furthermore, post-fertilization success and healthy offspring development rely on epigenetic reprogramming [20]. Similarly, epigenetic alterations may be responsible for age-related complications in fish oocytes.

Histone modifications are one of the epigenetic mechanisms that have been suggested to be involved in the oocyte aging process [15,16]. Histones are the most abundant protein components within the chromatin structure, where they form nucleoprotein complexes. They are the building blocks of the eukaryotic chromatin structure and are extensively conserved during evolution [21]. The reactions/processes associated with DNA occur at the nucleoprotein complex. A nucleosome is the basic unit of chromatin structure and consists of four different histones (H2A, H2B, H3, and H4) in the form of dimers that are collectively arranged as octamers [22]. The nucleosome possesses an N-terminal tail that is rich in arginine and lysine, and this is the site for post-translational modifications [23]. Histone modifications consist of different types that include acetylation and methylation of lysines and arginines, phosphorylation of serines and threonines, ubiquitylation, and ribosylation of lysines. Histone acetylation occurs more frequently than does any other modification [24,25]. Histone acetyltransferases (HATs) catalyse histone acetylation by transferring acetyl groups from acetyl coenzyme A (acetyl-CoA) to lysine residues within the core histones. Conversely, histone deacetylases (HDACs) catalyse the removal of acetyl groups. Together, HATs and HDACs regulate the acetylation status of histones. Histone acetylation and deacetylation play critical roles in controlling gene expression and several cellular functions [26,27]. Increased histone acetylation levels and histone acetyltransferase 1 (*hat1*) transcripts were observed during prolonged oocyte aging in mice [19], porcine [18], and goat [28]. The underlying pathways responsible for these alterations during fish oocyte aging remain unclear.

Fish exhibit benefits over other vertebrates in regard to the study of oocyte aging. The high number of oocytes produced by female fish and the diversity of reproductive modes make it possible to properly study the oocyte aging process. Specifically, studying oocyte aging at the molecular level may aid aquaculture and fisheries in managing egg quality. The current study employed Western blotting, supporting the reports of histone modifications during oocyte aging using immunohistochemistry in other vertebrates. This study was performed on common carp (*Cyprinus carpio*) because of synchronous ovulation in females and based on our previous satisfactory experience with the practical approaches. Additionally, common carp is one of the major aquaculture species in which the quality of eggs has been relatively well documented [29] and there is no need to sacrifice the experimental animal as it might be required in other vertebrates.

Histone modification dynamics were investigated during both in vivo and in vitro oocyte aging in common carp. Acetylation modifications at lysines 9 and 14 on histone H3 and at lysines 5, 8, 12, and 16 on histone H4 were analysed using specific antibodies. Additionally, a histone acetyltransferase activity assay was used to determine HAT activity in fresh and aged oocytes both in vivo and in vitro. The obtained results are the first to report histone modifications as an important epigenetic regulator during oocyte aging in fish and may also aid in the further development of assisted reproduction technology in higher vertebrates.

2. Results

2.1. Egg Quality Indices

The egg eyeing and hatching rates were significantly affected by post-ovulation and post-stripping oocyte aging, as presented in Table 1.

Table 1. Effects of in vivo and in vitro oocyte aging at 20 °C on the eyeing and hatching rates in common carp (mean ± SD). Means sharing a common alphabetical symbol do not differ significantly.

In Vivo Oocyte Aging								
HPO	**0**	**2**	**4**	**6**	**8**	**10**	**12**	**28**
Eyeing %	88 ± 9 ^{a}	91 ± 5 ^{a}	77 ± 16 ^{ab}	66 ± 30 ^{ab}	46 ± 25 ^{bc}	15 ± 11 ^{cd}	3 ± 4 ^{d}	0 ± 0 ^{d}
Hatching %	83 ± 16 ^{a}	88 ± 10 ^{a}	71 ± 21 ^{a}	59 ± 31 ^{ab}	31 ± 19 ^{bc}	6 ± 5 ^{c}	0.7 ± 1 ^{c}	0 ± 0 ^{c}
In Vitro Oocyte Aging								
HPO	**0**	**2**	**4**	**6**	**8**	**10**	**12**	**28**
Eyeing %	95 ± 4 ^{a}	94 ± 3 ^{a}	92 ± 3 ^{a}	84 ± 8 ^{a}	62 ± 6 ^{b}	40 ± 5 ^{c}		0 ± 0 ^{d}
Hatching %	94 ± 4 ^{a}	92 ± 1 ^{a}	88 ± 5 ^{a}	68 ± 15 ^{ab}	36 ± 22 ^{bc}	21 ± 9 ^{cd}		0 ± 0 ^{d}

HPO: Hours post-ovulation; HPS: Hours post-stripping.

2.2. Image Analysis of 2D AUT × SDS PAGE

In total, 37 protein spots matched among freshly ovulated and in vivo and in vitro aged oocyte samples (Supplementary Materials Files S1 and S2). The image analysis of 2D AUT × SDS PAGE revealed no significant differences between freshly ovulated oocytes and either in vivo or in vitro aged oocytes. However, at low significance ($p < 0.1$), spot numbers 80 (in vivo) and 113 (in vitro) were significantly different compared to those of the freshly ovulated oocyte samples. Spot number 80 exhibited a 1.3-fold decrease, while spot number 113 showed a significant 1.6-fold increase in oocyte aging.

2.3. Histone Acetylation during In Vivo and In Vitro Oocyte Aging

The acetylation of histones at H3K9, H4K5, and H4K8 did not significantly change during in vivo or in vitro oocyte aging (Figure 1A,B,D). Acetylation of H4K12 increased significantly in vitro but not during in vivo oocyte aging (Figure 1B). A comparison of histone acetylation at 8 and 28 h in vivo and in vitro did not reveal any significant changes (Figure 1C). Acetylation at H3K14 and H4K16 did not exhibit any signal in either freshly ovulated or in vivo and in vitro aged oocytes (Figure 1E). The efficiency of the related antibodies was confirmed by the presence of both modifications expressed in the mouse liver used as the positive control.

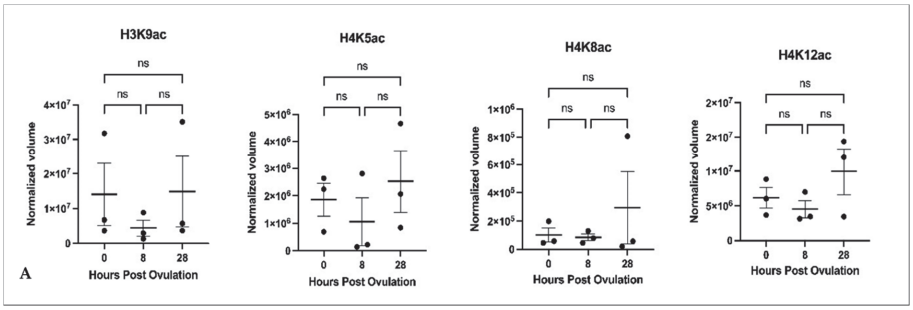

Figure 1. *Cont.*

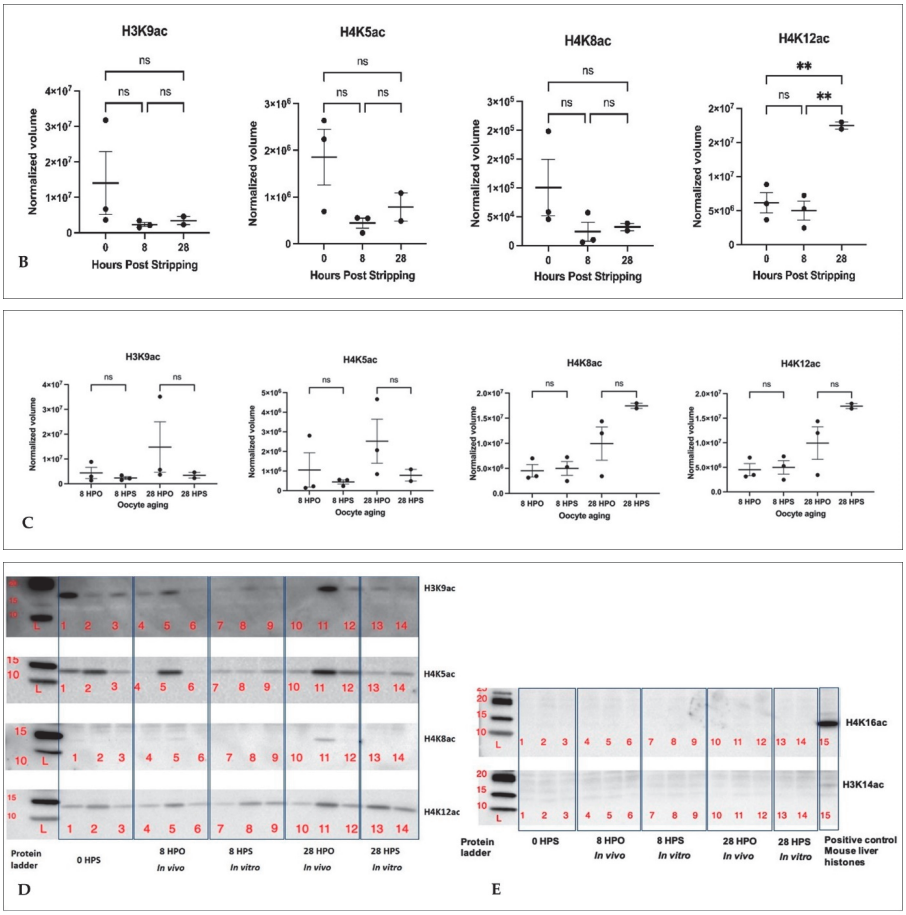

Figure 1. Effect of in vivo (**A**) and in vitro (**B**) oocyte aging on the acetylation of the selected histone lysines in common carp ($p < 0.05$, Tukey's multiple comparisons test; ns: non-significant; ** $p < 0.01$, mean ± SEM); (**C**) Comparison of histone acetylation at 8 and 28 h between in vivo and in vitro aging conditions. (**D**) Western blot images for H3K9ac, H4K5ac, H4K8ac and H4K12ac; (**E**) Western blot images for H3K16ac and H4K514ac including positive control (mouse liver histones). The numbers in red colour indicate the biological replicates as: fish 1 (1, 4, 7, 10), fish 2 (2, 5, 8, 11, 13), and fish 3 (3, 6, 9, 12, 14).

2.4. Histone Acetyltransferase Activity during In Vivo and In Vitro Oocyte Aging

Histone acetyltransferase activity was measured in freshly ovulated and in 8 h and 28 h in vivo and in vitro aged oocytes. HAT activity did not change significantly during in vivo or in vitro oocyte aging; however, a slight increasing trend was observed (Figure 2). A comparison of histone acetyltransferase activity at 8 and 28 h in vivo and in vitro also did not reveal any significant changes.

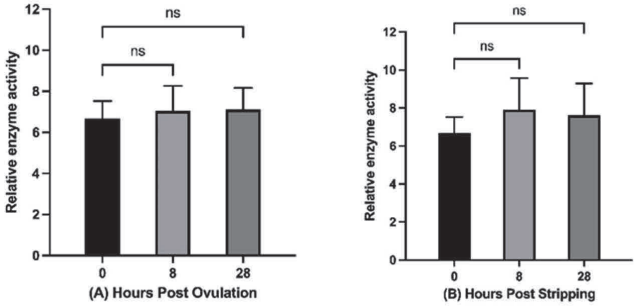

Figure 2. Effect of in vivo (**A**) and in vitro (**B**) oocyte aging at 20 °C on the histone acetyltransferase activity in common carp ($p < 0.05$, Tukey's multiple comparisons test; ns: non-significant; mean ± SEM).

3. Discussion

The competence of the oocyte to fertilise and develop into a normal embryo declines with increased post-ovulatory oocyte age that begins at ovulation and progresses constantly until fertilisation [1]. The mechanisms driving the oocyte aging process are not yet clear. Histone modifications are the most common and important epigenetic configuration that may contribute to age-associated defects in oocytes and the arising embryos. Establishment of chromatin structures is highly controlled by histone proteins. Many initial and essential functions, including cell cycle progression, DNA replication and repair, transcriptional activity, and chromosome stability, are associated with histone modifications [30–33].

In the present study, the results of global histone modification analysis suggest that no histone modifications are altered during post-ovulatory oocyte aging. However, at low significance ($p < 0.1$), spot numbers 80 (in vivo) (Supplementary Materials File S1) and 113 (in vitro) (Supplementary Materials File S2) were significantly different compared to those of the freshly ovulated oocyte samples. Spot number 80 exhibited a 1.3-fold decrease, while spot number 113 showed a significant 1.6-fold increase in oocyte aging. Post-translational modifications of H3 and H4 are much more widely identified than modifications on other histone proteins [34]. Acetylation modification of histones H3 and H4 has been reported in other vertebrates during post-ovulatory oocyte aging [15,16]. The key sites of lysine acetylation include two lysines on histone H3 (K9 and K14) and four lysines on histone H4 (K5, K8, K12, and K16), which are described as active transcription marks [34,35]. The acetylation and deacetylation of these N-terminal lysine residues both play a critical role in regulating chromatin condensation and folding, heterochromatin silencing, and transcription, and based on this they influence various cellular processes [27,36]. Therefore, we studied specific histone acetylations (H3K9ac, H3K14ac, H4K5ac, H4K8ac, H4K12ac, and H4K16ac) during in vivo and in vitro oocyte aging.

Among the selected histone acetylation modifications examined in this study, H3K9ac, H4K5ac, and H4K8ac exhibited no significant differences during in vivo and in vitro oocyte aging. However, Huang, et al. [19] reported no signal for histone acetylation at H3K9 and H4K5 and increased H4K8ac during in vivo and in vitro mouse oocyte aging. In contrast, Xing et al. [37] observed an increase in H3K9ac in aged mouse oocytes and attributed the difference to the different implemented oocyte culture media and antibodies. In our study, H3K14 and H4K16 were not acetylated in either fresh or aged common carp oocytes. Liu et al. [38] also did not detect a signal for H3K14ac in fresh oocytes; however, this signal was detected in aged mouse oocytes. A gradual increase in H3K14ac was reported during oocyte aging in mice [19]. In vivo and in vitro aged mouse oocytes displayed a signal for the acetylation of H3K14 in 76% and 75% of the oocytes, respectively, and no signal was observed in freshly ovulated oocytes [38]. All of the investigated histone

acetylation modifications in our study have been reported to be species-specific and oocyte stage dependent [34]. Accordingly, the results of our study revealed undetected H3K14ac and H4K16ac in common carp MII oocytes. In future studies, it would be of interest to investigate the dynamics of different histone modifications during fish oocyte maturation and embryo development.

In the current study, the acetylation on H4K12 was increased significantly at 28 HPS. This is in accordance with previous findings in other vertebrates. Immunofluorescence detection of H4K12 acetylation in mouse oocytes revealed an increased signal during 5 and 10 h of aging [19]. All 12 h-aged mouse oocytes exhibited signals for the acetylation on H4K12, while no signal was detected in freshly ovulated oocytes [38]. Increased fluorescence signals have been reported for H4K12ac in 24 h-aged porcine and mouse oocytes [18,39]. Cui et al. [18] suggested that increased H4K12ac in 28 h-aged porcine oocytes is associated with increased oxidative stress within the ooplasm. The observed increase in H4K12ac levels in oocytes aged for 28 h post-stripping in the current study may therefore be a consequence of increased oxidative stress at this time point; however, a previous study examining common carp indicated that oxidative stress is not likely to be the main initiator of post-ovulatory aging in common carp oocytes, at least up to 14 h in vivo and 10 h in vitro [40]. Moreover, the observed late onset of hyperacetylation of H4K12 suggests the possible optimisation of egg storage in common carp. H4K12ac is essential for centromere protein A deposition into centromeres [41], a process that is required for accurate chromosome segregation during cell division [42]. Furthermore, H4K12ac has been suggested to play a critical role in loosening chromatin structures during DNA replication [43]. Post-ovulatory oocyte aging may be one of the factors that spontaneously release the zygotic clock to thereby trigger molecular pathways [44].

The zygotic clock is a molecular clock that initiates cascades of biochemical processes that occur post-fertilization or after egg activation [45]. Post-translational histone modifications are among the mechanisms underlying zygotic genome activation [46]. Histone acetylation is a marker of active transcription that is projected to increase during the maternal to zygotic transition and is linked to the activation of genes [46,47]. Therefore, the observed hyperacetylation of H4K12 in aged oocytes in this study may be due to the spontaneous activation and ticking of the zygotic clock. Some studies have also reported dynamic changes in transcripts and in protein abundance during post-ovulatory oocyte aging that are known to be transcriptionally inactive [13,28,39,48]. While post-ovulatory oocyte aging does not lead to complete zygotic genome activation, it may trigger some of the pathways and partially explain the functional consequences of oocyte aging. Until now, few studies have compared the oocyte aging in vivo and in vitro conditions. The comparison of histone acetylation at 8 and 28 h in this study did not reveal any significant changes between in vivo and in vitro aging. This is in accordance with the previous finding by Zhang et al. [49], who reported no significant difference in the decreased expression of selected histone deacetylases transcripts during 24 h in vivo and in vitro oocyte aging in mouse.

The acetylation–deacetylation switch depends upon different physiological conditions, and this switch is achieved through the action of HATs and HDACs [20]. The HAT activity in the current study tended to increase during oocyte aging, although the difference was not statistically significant. *Hat1* transcript levels have also been reported to increase during porcine and goat oocyte aging [18,28]. The mRNA levels of the histone deacetylases *sirt1*, *sirt2* and *sirt3* were downregulated in mouse oocytes that were aged in vivo and in vitro [49]. Another study reported the downregulation of a gene responsible for histone deacetylation (*hdac2*) during maternal oocyte aging in mice [50]. These altered expressions of key epigenetic modulators in aged oocytes may lead to abnormal histone acetylation.

4. Materials and Methods

The histone modification dynamics during in vivo and in vitro oocyte aging in common carp was assessed through three experiments as: (I) egg storage and quality assessment, (II) histone modifications and (III) histone acetyltransferase activity assay.

4.1. Egg Storage and Quality Assessment

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

4.1.1. Experimental Fish Preparation

The broodfish preparation and artificial reproduction processes were performed according to Samarin et al. [51] and Samarin et al. [40]. Briefly, the experimental fish were treated with a gradual increase in water temperature from 16 °C to 20 °C. The carp pituitary hormone was used to stimulate ovulation and spermiation according to the method of Horvath and Tamas [52]. The experiment was conducted using six females for both the in vivo and in vitro experiments.

4.1.2. In Vivo and In Vitro Oocyte Aging

Oocytes from females were individually incubated in vivo for 28 h post-ovulation (HPO) and in vitro for 28 h post-stripping (HPS) at 20 °C. The stored oocytes in vivo were stripped and fertilised at the time of ovulation (0 HPO) and then at 2 h intervals up to 12 HPO and also at 28 HPO. The stored oocytes in vitro were fertilised at the time of stripping (0 HPS) and then at 2 h intervals up to 10 HPS and also at 28 HPS. The in vivo and in vitro egg storage conditions were determined according to Samarin et al. [40].

4.1.3. Artificial Fertilisation and Egg Developmental Success

Eyeing and hatching rates were recorded as the egg quality indices. Three days post-fertilisation, the ratio of the number of eyed eggs to the number of initial inseminated eggs was used to calculate eyeing percentages. Hatching rates were measured after six days of fertilisation using the number of hatched larvae to the number of initially inseminated eggs. All of the steps for artificial fertilisation, removing egg stickiness, incubation, and assessment of egg developmental success were performed according to Samarin et al. [51].

4.2. Histone Modifications

The global histone modifications and specfic histone acetylation were investigated as bellow:

4.2.1. Sample Collection for Histone Modification Analysis

Approximately 1 g of oocytes was sampled from individual females separately at each HPO and HPS. The collected samples were placed into cryotubes (Thermo Fisher Scientific, Waltham, MA, USA), labelled, frozen in liquid nitrogen, and stored at −80 °C for further investigation.

4.2.2. Histone Isolation

Histone isolation was performed for samples collected at 0, 8, and 28 HPO and HPS. The isolation procedure was performed according to Wu et al. [53] with some modifications. Briefly, 150 mg of frozen oocyte samples were resuspended and homogenised in a 1.5 mL tube containing 400 µL of homogenisation buffer (10 mM Tris-HCl; 1 mM $MgCl_2$; pH 7.5) and 20 µL of 5% digitonin (D141, Sigma-Aldrich, St. Louis, MO, USA). After centrifugation, the supernatant was discarded, and 1 mL of salt wash buffer (10 mM Tris-HCl; 1 mM $MgCl_2$; 0.4 M NaCl; pH 7.5) was added to the pellet and incubated on ice for 15 min. Samples were then centrifuged, and the pellets were used for acid extraction of histones. Acid-extracted histones were precipitated using saturated trichloroacetic acid (T6399, Sigma-Aldrich, St. Louis, MO, USA). The air-dried histone pellets were then dissolved in 50 µL of Milli-Q

water and stored at −20 °C for further analysis. The quality and quantity of isolated histones were both assessed by separating 5 µL of the histone on 4–15% Criterion™ TGX™ Precast Midi Protein Gels (Bio-Rad, Hercules, CA, USA).

4.2.3. Separation of Histone Modifications

The 2D AUT × SDS PAGE (acetic acid-urea-Triton sodium dodecyl sulfate polyacrylamide gel electrophoresis) assay was used to separate the isolated histones. The Mini Trans-Blot® Cell system (Bio-Rad, Hercules, CA, USA) was used for both the first- and second-dimension separations. The gel electrophoresis procedure was adopted from Shechter et al. [54] and Green and Do [55]. In summary, histone samples were separated on 15% AUT-PAGE, and the running buffer (5% acetic acid) was used for electrophoresis at 200 V for 140 min. Thereafter, the AUT gel target lanes were cut individually and carefully placed on top of the SDS-PAGE gel (4–15%). The stacking gel was poured around the AUT gel lane and allowed to polymerise. The second-dimension gel was run at a constant voltage of 150 V for 90 min, and this was followed by Coomassie staining. Gel images were recorded using the ChemiDoc™ XRS$^+$ system (Bio-Rad, Hercules, CA, USA). Image analysis of 2D AUT × SDS PAGE was performed using Prodigy SameSpots version 1.0 software (Nonlinear Dynamics, Newcastle, UK).

4.2.4. Immunodetection of Specific Histone Acetylation

For the analysis of specific histone acetylation modifications, isolated histone samples were separated on 4–15% Criterion™ TGX™ Precast Midi Protein Gels (Bio-Rad, Hercules, CA, USA). The isolated histone proteins were transferred to a 0.2 µm nitrocellulose membrane using the Trans-Blot Turbo Transfer Pack (Bio-Rad, Hercules, CA, USA) and the Trans-Blot Turbo Blotting System (Bio-Rad, Hercules, CA, USA) at 2.5 A and 25 V for 7 min. The membranes were then blocked for 1 h at room temperature in 5% bovine serum albumin (BSA) in Tris-buffered saline-Tween 20 (TBST) buffer. Thereafter, the blot was incubated overnight at 4 °C with the primary antibody diluted in blocking solution according to the manufacturer's instructions (Abcam, Cambridge, UK). The selected specific histone acetylation modifications were studied using the following antibodies: Anti-AcH3K9, ab10812; Anti-AcH3K14, ab52946; Anti-AcH4K5, ab51997; Anti-AcH4K8, ab15823; Anti-AcH4K12, ab46983; Anti-AcH4K16, ab109463 (Abcam, Cambridge, UK). Separate blots were used to analyse the modifications. After incubation with the primary antibody, the blots were washed in TBST buffer and incubated with the secondary antibody that consisted of goat anti-rabbit IgG HRP conjugate (12-348, Millipore, Billerica, MA, USA) diluted 1:8000 in 1% BSA prepared in TBST buffer for 1 h at room temperature. After washing in TBST, the blots were developed using an ECL kit (1705061, Bio-Rad Laboratories, Hercules, CA, USA).

4.3. Histone Acetyltransferase Activity Assay

HAT activity was measured using a Histone Acetyltransferase Activity Assay Kit (Colorimetric) (ab65352, Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, approximately 50 mg of the oocyte samples at 0, 8, and 28 HPO and HPS were homogenised in 0.1% Triton X-100 on ice and then centrifuged at 10,000× *g* for 5 min at 4 °C. The supernatant was used for histone acetyltransferase activity assays. The total protein concentration was estimated using the Bio-Rad protein assay kit (500-0001, Bio-Rad Laboratories, Hercules, CA, USA). Fifty micrograms of protein extract were incubated with HAT substrate I, HAT substrate II, and NADH-generating enzyme in HAT assay buffer for up to 4 h at 37 °C. An ELISA plate reader (Synergy 2, BioTek Instruments, Winooski, VT, USA) was used to measure the absorbance at 440 nm. The active nuclear extract was used as a positive control and a standard. All measurements were performed in duplicate.

4.4. Statistical Analysis

The 2D AUT × SDS PAGE images were normalised and analysed using the Prodigy SameSpots software (Nonlinear Dynamics, Newcastle, UK) to determine differences in

staining intensities among gels. The protein spots exhibiting a >1.5 average fold difference in spot volume were recognised as differentially expressed between the fresh and aged oocyte samples. Western blot images were normalised using the corresponding stain-free gel image and quantified using Image Lab 6.1.0 (Bio-Rad Laboratories, Hercules, CA, USA) software. The quantified Western blot images and histone acetyltransferase activity data were analysed using GraphPad Prism 9.1.0 (GraphPad Software, San Diego, CA, USA). Tukey's multiple comparisons test was used to determine the differences in histone acetylation and histone acetyltransferase activity assays during oocyte aging. Additionally, histone acetylation and histone acetyltransferase activity were compared between oocytes aged 8 and 28 h in vivo and in vitro. Differences were considered significant at $p < 0.05$.

5. Conclusions

This study confirmed the presence of acetylation markers on H3K9, H4K5, H4K8, and H4K12 in common carp metaphase II oocytes. There was no evidence of acetylation at H3K14 and H4K16 in either fresh or aged oocytes. Furthermore, an increased acetylation pattern of H4K12 was observed during 28 h of in vitro oocyte aging in common carp. These findings highlight the dynamics of other histone modifications and epigenetic regulators during fish oocyte aging. Furthermore, it will be of interest to investigate the genomic regions that are associated with the hyperacetylation that occurs due to fish oocyte aging.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the EU-harmonized Animal Welfare Act of the Czech Republic and approved by the expert committee of the Institutional Animal Care and Use Committee (IACUC) of the University of South Bohemia, Czech Republic. According to the Czech National Directive, the unit is licensed (No. 16OZ19179/2016-17214) (Law against Animal Cruelty, No. 246/1992). The co-authors of this study were involved in the manipulation of fish and hold certificates giving them the right to work with laboratory animals according to Section 15d, paragraph 3, of Act No. 246/1992 Coll. Any transfer and manipulation of fish was performed after anaesthesia to minimise the associated stress and ensure their welfare.

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CHAPTER 4

IN VITRO POST-OVULATORY OOCYTE AGEING AFFECTS H4K12 ACETYLATION PATTERN AND HISTONE ACETYLTRANSFERASE ACTIVITY IN GRASS CARP *CTENOPHARYNGODON IDELLA*

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My share on this work was 30%.

In vitro post-ovulatory oocyte ageing affects H4K12 acetylation pattern and histone acetyltransferase activity in grass carp *Ctenopharyngodon idella*

Azin Mohagheghi Samarin[1*], Azadeh Mohagheghi Samarin[1], Swapnil Gorakh Waghmare[1], Marianne Danielsen[2,3,4], Hanne Søndergård Møller[2], Tomáš Policar[1], Otomar Linhart[1], Trine Kastrup Dalsgaard[2,3,4]

[1] South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Zátiší 728/II, 389 25 Vodňany, Czech Republic
[2] Department of Food Science, Aarhus University, Agro Food Park 48, 8200 Aarhus N, Denmark
[3] CiFOOD, Aarhus University Centre for Innovative Food Research, 8000 Aarhus C, Denmark
[4] CBIO, Aarhus University Centre for Circular Bioeconomy, 8000 Aarhus C, Denmark

** Corresponding author: Azin Mohagheghi Samarin*
E-mail: mohagheghi@frov.jcu.cz

Abstract

Delayed fertilization leads the way to post-ovulatory ageing of oocytes which reduces the oocyte developmental competence. Not much is known about the molecular processes during fish oocyte ageing. The current study investigated the functional consequences of oocyte ageing in grass carp *Ctenopharyngodon idella*. In addition, the abundance of post-transcriptionally modified histones (acetylation of H3K9, H3K14, H4K5, H4K8, H4K12 and H4K16) were analysed during oocyte ageing. Matured oocytes were aged *in vitro* for 4 h post-ovulation and analyzed by western blotting for selected post translational modification of histones. In addition, histone acetyltransferase activity, which is the determinant of histone acetylation, was also investigated. Results showed significantly reduced viability rates during oocyte ageing. Furthermore, post-ovulatory ageing led to decreased levels of H4K12 acetylation. The activity of Histone acetyltransferases as an important regulator of histone acetylation modification increased significantly only after ageing for 30 h *in vitro*. Considering all, post-ovulatory ageing affects epigenetic modifications of H4K12 which may partly explain the failures associated with reduced egg viability rates and emerging embryos.

Keywords: *Ctenopharyngodon idella, Egg viability, Histone acetylation, Oocyte, post-ovulatory ageing*

1. Introduction

The ability of the oocyte to develop to normal embryo may decline with increased post ovulatory oocyte age which starts at ovulation and makes a progress continuously (Petri et al., 2020). Post ovulatory ageing may occur by the prolonged residence of the oocyte in the oviduct or body cavity *in vivo* or *in vitro* in the culture media before insemination in assisted reproductive technologies (ART) (Samarin et al., 2019). In fish, prolonged residence of the oocyte before fertilization might occur due to delays in egg stripping or spawning naturally. Post-ovulatory ageing is known to reduce the oocyte quality and can lead to defects in emerged embryo developmental competence. Additionally increasing time between ovulation and fertilization enhance cytogenetic abnormalities (Petri et al., 2020) and lead to unhealthy status in later life of the offspring (Tarin et al., 2002). Hence, interests are rising to investigate

molecular mechanisms underlying these consequences in aged oocytes which are still poorly understood. Previous studies of different model organisms such as bovine, porcine, mice, and human have suggested mechanisms that are likely involved in the ageing of oocytes. These include oxidative damage (Lord and Aitken, 2013), mitochondrial dysfunction (Takahashi et al., 2011), chromosome anomalies (Mailhes et al., 1998), deadenylation of poly(A) tail length of maternal effect genes (Dankert et al., 2014) and epigenetic changes (Huang et al., 2007; Ge et al., 2015; Trapphoff et al., 2016). It is also accepted that apoptosis is the driving force behind oocyte fertilization loss with ageing.

Epigenetic mechanisms are key regulators of gene transcription, with particular significance in responses to altered environmental signals (Jaenisch and Bird, 2003). Post-ovulatory ageing as an environmental factor may lead to altered epigenetic modification in the aged oocytes. Histone modifications are among the most important and common epigenetic configurations which play a key role in post-fertilization success and early embryo development (Gao et al., 2010; Beaujean, 2014; Rollo et al., 2017; Hanna et al., 2018; Liu et al., 2018). Histones are globular proteins which are wrapped around by DNA in nucleus (Kouzarides, 2007). The tail part of histones could be involved in acetylation and ubiquitination of lysine residues, phosphorylation of serines, and methylation of lysine and arginines (Berger, 2007) which are described as post-translational modification of histones (Kouzarides, 2007). The acetylation of histones happens more frequently than any other modifications (Gavazzo et al., 1997). The levels of acetylation and deacetylation in histones have important roles in regulation the gene expression and various cellular functions (Kurdistani and Grunstein, 2003). The extent of histone acetylation is controlled by proteins, such as histone acetyltransferases (HATs and myst) and histone deacetylase (HDACs) (Gu et al., 2010). Alterations in their expression during oocyte ageing can directly affect epigenetic related modifications. Acetylation modification which occurs on histone H3 and H4 has been reported in other specious during post-ovulatory oocyte ageing (Liang et al., 2012, Ge et al., 2015). Increased histone acetylation levels (Huang et al., 2007; Cui et al., 2011) and *hat1* transcript (Zhang et al., 2013) were observed during prolonged oocyte ageing. Studies on higher vertebrates have shown that post-ovulatory oocyte ageing induces modifications on histones (Petri et al., 2020). We previously observed significant increase in acetylation of H4K12ac during *in vitro* oocyte ageing in common carp (*Cyprinus carpio*) (Waghmare et al., 2021b).

To approach closer to the underlying molecular mechanisms of post-ovulatory oocyte ageing and to have a better picture of pathways involved in the progress of this process, the present study investigated dynamics of histone acetylation in grass carp oocytes during prolonged culture. The high number of oocytes produced and easy manipulation of oocytes *in vitro* as well as intrinsic nature of external fertilization in fish make the study of oocyte ageing in fish effective and more convenient once comparing with other model organisms. The technical limitations in higher vertebrates due to limited number of cells before the stage of early blastula is becoming a considerable advantage in fish as the duration of this stage is longer and the number of cells is more compared with other model organisms.

Grass carp is one of the major freshwater farmed fish species with the largest aquaculture production globally (FAO, 2020). It has also been intentionally widely introduced worldwide to use as biological control of aquatic vegetation (Bozkurt et al., 2017; Jones et al., 2017). The availability of genome information has made it easy to investigate the biology of grass carp in-depth (Wang et al., 2015; Chen et al., 2017). However, being the most extensive aquaculture species, the egg quality in grass carp has been not profoundly studied at the molecular level. Comprehensive knowledge of egg quality could lead to better understanding and management of the key aquaculture fish species.

The current study investigated the developmental capacity of oocytes that where aged *in vitro* for up to 4 h after ovulation. The ability to develop to the embryo stage was investigated, and intensity of specific histone acetylation were analyzed in fresh and post-ovulatory aged oocytes. The highly conserved lysine that are vulnerable to acetylation modification are known as (K5, K8, K12 and K16) on H4 and (K9 and K14) on H3. Therefore, the important histone acetylation marks on histone H3 at lysines (K) 9 and 14; on histone H4 at lysines (K) 5, 8, 12 and 16 were investigated using specific antibodies. In addition, histone acetyltransferase activity, which is the determinant of histone acetylation, was also investigated. Provided results will uncover a part of epigenomics in oocyte ageing and direct to more in-depth studies. Specifically, studying oocyte ageing at the molecular level could contribute to both basic research to understand fundamentals and applied research in aquaculture to delay oocyte ageing. Furthermore, assisted reproduction technology in other vertebrates could also be reinforced with the current findings.

2. Methods

2.1. Animal experiment, *in vitro* oocyte storage, artificial fertilization and egg developmental success

The broodfish preparation and artificial reproduction processes were performed as followings. Wild grass carp broodfish were captured from the earthen ponds at Nove Hrady, Czech Republic when the daily water temperature reached to the mean of 20±1 °C. The fish were then transferred to the Experimental Fish Culture Facility at the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic. The fish were placed into an indoor recirculating aquaculture system (2,880 l capacity tanks) under the following conditions: pH 7.0±0.2, oxygen saturation: 75±10%. The photoperiod was adjusted at 14-hours light:10-hours dark. The water temperature was gradually increased from 20 to 25 °C within 5 days (1 °C/ day). Ovulation was induced with a muscular injection Carp Pituitary Extract CPE (0.3 mg kg^{-1} body weight) followed by a second injection with CPE (3.5 mg kg^{-1} body weight) after 12 h. Simultaneously during the second injection of females, a single muscular injection of CPE (2 mg kg^{-1} body weight) was given to males to induce spermiation. Prior to hormonal injection and examining ovulation and stripping, fish were anesthetized with a 0.03 ml/L clove oil water bath. The experiment was conducted using five females. Oocytes from females were individually incubated *in vitro* for 30 h post-stripping (HPS) at 25 °C. The stored oocytes were fertilized at the time of stripping (0 HPS) and then at 30 min, 1, 2 and 4 HPS. To examine the embryo survival rates, 200 eggs were used from each female at each time point; that is, in total, 5,000 embryos were evaluated for developmental success. Embryo survival rates were measured 24 h after fertilization using a stereomicroscope (Nikon SMZ745T, Japan).

2.2. Histone acetylation dynamics

2.2.1. Sample collection for histone modification analysis

At each selected post-stripping time point, approximately 1 g of oocytes were sampled separately from individual fish into cryotubes (Thermo Fisher Scientific, USA). The collected samples were frozen in liquid nitrogen and stored at -80 °C for further investigation.

2.2.2. Histone isolation

The oocyte samples collected at 0, 1 and 4 HPS were selected for the histone isolation. Isolation was performed the same as previously reported in common carp (Waghmare et al., 2021). In brief, the oocyte samples (150 mg) were homogenised in a 1.5 ml tube containing 400 µL of homogenisation buffer which included 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$. During homogenisation, 20 µL of 5% digitonin (D141, Sigma-Aldrich, Missouri, USA) were added. The homogenate was then centrifuged, and the supernatant aspired, and the pellet was incubated with 1 ml salt wash buffer on ice for 15 min. The pellets washed in salt was buffer were then resuspended in an acid extraction solution, and histones were then precipitated using saturated trichloroacetic acid (T6399, Sig-ma-Aldrich, Missouri, USA). The obtained histone pellets were then air-dried and redissolved in 50 µL of Milli-Q water. The histone samples were then stored at -20 °C for further analysis. The 5 µL of the dissolved histone samples were separated on 4–15% Criterion™ TGX Stain-Free™ Protein Gel (Bio-Rad, USA) to assess the quality and quantity.

2.2.3. Immunodetection of specific histone acetylation

Isolated histone samples were first separated on the SDS-PAGE (4–15% Criterion™ TGX Stain-Free™ Protein Gel, Bio-Rad, USA). Following SDS-PAGE, histone samples were electronically transferred to nitrocellulose membrane (0.2 µm size, Trans-Blot Turbo Transfer Pack, Bio-Rad, USA) using the Trans-Blot Turbo Blotting System (Bio-Rad, USA) at 2.5 A and 25 V for 7 minutes. Membranes were blocked by incubation with 5% bovine serum albumin (BSA) prepared in Tris-buffered saline-Tween 20 (TBST) buffer for 1 hour at room temperature. The blocked membranes were then incubated overnight at 4 °C with the primary antibody diluted in blocking solution (5% w/v BSA-TBST buffer), as specified by the manufacturer (Abcam, Cambridge, UK). The primary antibodies used in the current study include anti-AcH3K9, ab10812; anti-AcH3K14, ab52946; anti-AcH4K5, ab51997; anti-AcH4K8, ab15823; anti-AcH4K12, ab46983; and anti-AcH4K16, ab109463 (Abcam, Cambridge, UK). Membranes were then washed and incubated with the secondary antibody (goat anti-rabbit IgG HRP conjugate; 12-348, Millipore, USA) diluted 1:8,000 in blocking solution (1% w/v BSA-TBST buffer). After washing in TBST, the reacted secondary antibodies were detected using an ECL kit (1705061, Bio-Rad Laboratories, USA). To test the efficiency of primary antibodies, histones isolated from the mouse liver were used as a positive control. The non-specific binding of the secondary antibody (negative control) was tested by eliminating the primary antibody during the immunodetection protocol.

2.3. Histone acetyltransferase activity assay

The commercially available kit, Histone Acetyltransferase Activity Assay Kit (Colorimetric) (ab65352, Abcam, Cambridge, UK), was used to assess the HAT activity. All the steps of the HAT activity assay were performed according to the manufacturer's instructions. The oocyte samples at 0, 1, 4 and 30 HPS were used in the HAT activity assay. All subsequent steps were performed either on ice or at 4 °C. In brief, the oocyte samples (~50 mg) were homogenised in 0.1% Triton X-100 and centrifuged at 10,000 g for 5 min. The assay was performed using the supernatant, and the total protein quantification was determined using the Bio-Rad protein assay kit (500-0001, Bio-Rad Laboratories, USA). The assay reaction was performed by incubating the 50 µg of the isolated protein, HAT substrate I, HAT substrate II, and NADH-generating enzyme in the HAT assay buffer. Positive control and the standard were provided

with the kit as an active nuclear extract. The assay plate was incubated at 37 ºC for up to 4 h. All the reactions and measurements were carried out in duplicates. The absorbance was recorded at 440 nm using an ELISA plate reader (Synergy 2, BioTek Instruments, USA).

2.4. Statistical analysis

The statistical analysis was performed using GraphPad Prism 9.1.0 (GraphPad Software, San Diego, California USA). The embryo survival rates were evaluated using an ANOVA followed by Duncan's multiple range test. The corresponding stain-free gel image was used normalise the Western blot images using Image Lab 6.1.0 (Bio-Rad Laboratories, USA) software. The western blots were quantified using Image Lab 6.1.0. The differences in histone acetylation and histone acetyltransferase activity were assessed by Tukey's multiple comparisons test. Differences were considered significant at $p<0.05$.

3. Results

3.1. Egg viability

The embryo survival rate remained unchanged up to 30 minutes post-stripping. Then survival values reduced considerably at 1 hour post stripping (HPS) and did not change significantly until 4 HPS which measured 1.6% as the lowest embryo survival rate determined in the present study (Fig. 1).

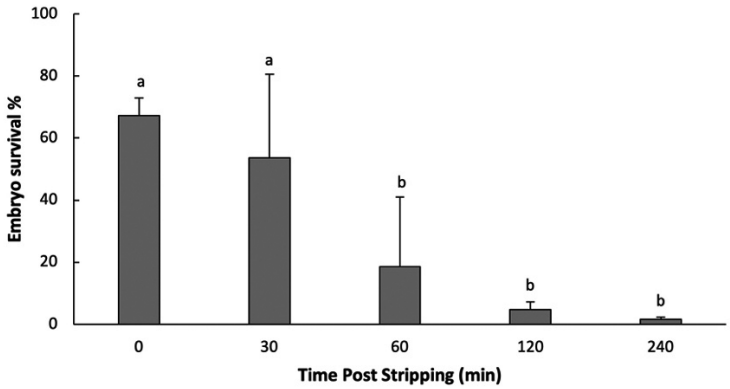

Figure 1. *Effect of post-stripping ova ageing on the embryo survival rates in grass carp (mean ± SD). Means sharing a common alphabetical symbol do not differ significantly.*

3.2. Histone acetylation during oocyte ageing

The histone H3K9ac, H4K5ac, and H4K8ac did not change significantly among fresh, 1 and 4 HPS aged oocytes. (Fig. 2). The histone H4K12ac decreased significantly in oocytes aged for 1- and 4- HPS. Neither fresh nor aged oocytes exhibited histone H3K14ac or H4K16ac signals. The histone samples isolated from mouse liver were used as a positive control to show the efficiency of anti-H3K14ac and anti-H4K16ac antibodies.

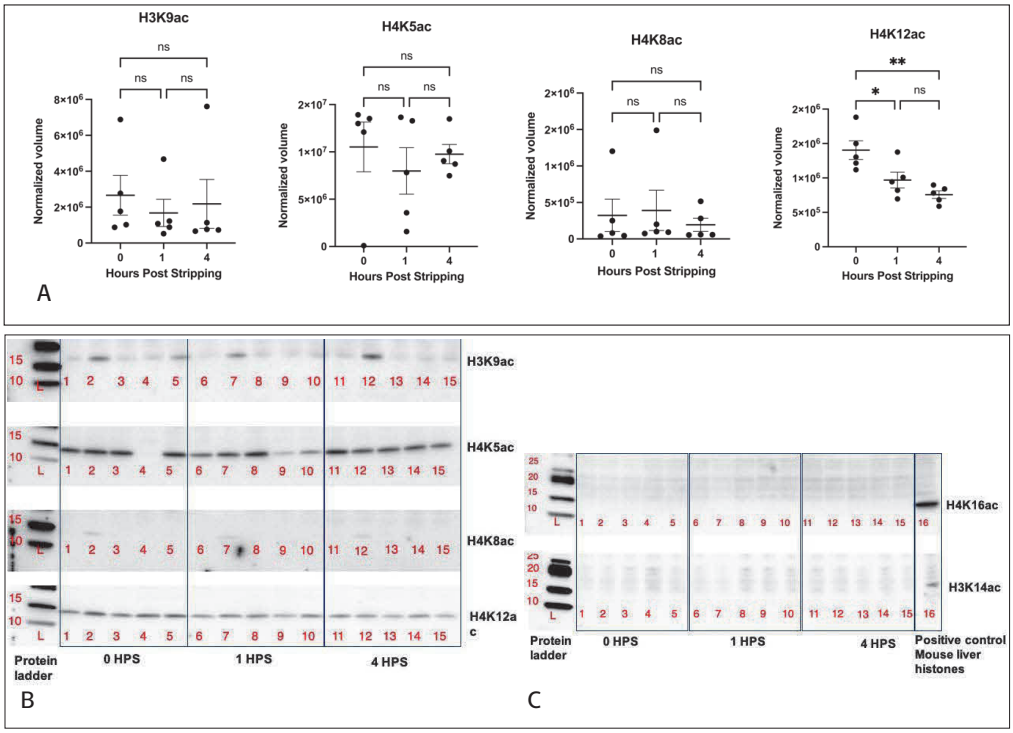

Figure 2. *(A) Effect of in vitro oocyte ageing on the acetylation of the selected histone lysines in grass carp (Tukey's multiple comparisons test; ns: non-significant; * p<0.05, and ** p<0.01, mean ± SEM); (B) Western blot images for H3K9ac, H4K5ac, H4K8ac and H4K12ac; (C) Western blot images for H3K16ac and H4K514ac including positive control (mouse liver histones). The number in the red colour indicate the biological replicates as: fish 1 (1, 6, 11), fish 2 (2, 7, 12), fish 3 (3, 8, 13), fish 4 (4, 9, 14) and fish 5 (5, 10, 15).*

3.3. Histone acetyltransferase activity during oocyte ageing

Histone acetyltransferase activity was measured in freshly ovulated and in 1-, 4- and 30-hours *in vitro* aged oocytes. The HAT activity increased significantly at 30-hours post-stripping compared to freshly ovulated,1- and 4-hours *in vitro* aged oocytes (Fig. 3). The differences in HAT activity among freshly ovulated and in 1- and 4-hours post-stripping aged oocytes were not significant.

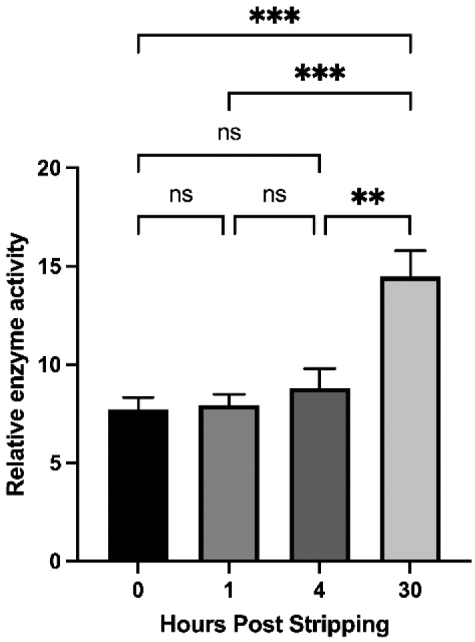

Figure 3. *Effect of in vitro oocyte ageing on the histone acetyltransferase activity in grass carp (p<0.05, Tukey's multiple comparisons test; ns: non-significant; ** p<0.01, *** p<0.001, mean ± SEM).*

4. Discussion

Oocyte integrity is crucial for the support of normal embryo development (Keefe et al., 2015; Fair, 2018). Ageing of oocytes following ovulation is an unfavourable and unavoidable process and known as the most determinant factor of the oocyte quality. The optimal window of time for fertilization varies in different organisms and in turns of fish very much differs in between species (Samarin et al., 2015b). Post-ovulatory oocyte ageing leads to decreased fertilization capacity and declines the developmental potential of emerged embryos. The current study shows that *in vitro* post-ovulatory ageing of grass carp oocytes for up to 30 minutes post stripping exhibited no effect on derived embryo survival rates. Survival values reduced considerably at 1 HPS and did not change significantly until 4 HPS which measured 1.6% as the lowest embryo survival rate determined in the present study (Fig. 1). These results are in line with previous findings by Safarzadenia et al. (2013) who observed that egg viability rates significantly decreased up to 3 hours post fertilization during the storage of grass carp oocytes at 20 °C. These authors did not store grass carp oocytes in ovarian fluid as current study did but, stored oocytes in 2 different artificial medias including grass carp artificial coelomic fluid and Dettlaff extender (111.3 mM NaCl+3.3 mM KCl+2.1 mM $CaCl_2$+23.8 mM $NaHCO_3$). They reported that the artificial storage media has significant effects on eyeing and hatching rates during post ovulatory ageing of grass carp oocytes. *In vitro* oocyte ageing of grass carp at 9 and 22 °C showed the fertilization percentage of 87% determined 1 h post spawning which is in contrary with the results of current study. At 5 h after the spawning fertilization rates decreased to 6.5% and 16.6% for the eggs stored at 9 and 22 °C respectively (Zlabek and Linhart, 1987).

Yet the proceeding of oocyte ageing, and its underlying molecular mechanisms has been poorly understood. Epigenetic alterations are categorized as primary hallmarks of

ageing which are unambiguously negative and are causes of damage (Lopez-Otin et al., 2013) Thus, epigenetic modification in post-ovulatory aged oocytes may be altered. This epigenetic configuration mostly includes methylation of CpG islands and methylation/ acetylation of histones (Li, 2002; Shiota and Yanagimachi, 2002). Post-translational histone modifications can modify chromatin activity and therefore have important roles in regulation the gene expression and various cellular functions which in turn controls the arising embryo development (Peterson and Laniel, 2004; Cedar and Bergman, 2009). In the present study dynamics of histone acetylation as important epigenetic regulator which might probably be responsible for effects of postovulatory ageing in grass carp oocytes were investigated. The highly conserved lysine that are vulnerable to acetylation modification are known as K5, K8, K12 and K16 on H4 and K9 and K14 on H3. To explore the involvement of histone acetylation in the process of oocyte ageing, probable changes in the acetylation patterns of various lysine residues in histones H3 and H4 during postovulatory ageing of grass carp oocyte was examined.

In the present study, acetylation levels of H3K9, H4K5 and H4K8 as important epigenetic regulators was not affected by *in vitro* oocyte ageing. Similarly, in common carp oocytes the acetylation of histones at H3K9, H4K5, and H4K8 did not significantly change during post-ovulatory aging both *in vivo* and *in vitro* (Waghmare et al., 2021b). This observation suggests the stable and conserved nature of H3K9, H4K5 and H4K8 acetylation marks in common carp and grass carp metaphase II (MII) oocytes. Studies in mouse reported no signal for H3K9 and H4K5 acetylation during *in vivo* and *in vitro* oocyte ageing (Huang et al., 2007). These authors however, reported a gradual increase in the acetylation levels of H4K8 (Huang et al., 2007). An increase in H3K9ac however, was reported by Xing et al. (2021) in post-ovulatory aged mouse oocytes. Another study showed that *in vitro* post-ovulatory ageing induces alterations in H3K9 tri-methylation on mouse oocyte and the arising two-cell embryos (Petri et al., 2020). In the future it would be of interest to investigate the dynamics of histone methylation during prolonged culture of fish oocytes and embryo development. Among the selected histone modifications analyzed in this study, H3K14ac and H4K16ac was not expressed either in fresh or aged oocytes. The observed absence of H3K14ac and H4K16ac in fresh or aged oocytes is identical to that of common carp oocytes during the progress of post-ovulatory ageing (Waghmare et al., 2021b). Therefore, as both the studied fish species belong to the same family Cyprinidae, the mechanisms of regulation histone modifications might be conserved. These results however are in contrast with the obtained results by Huang et al. (2007) who reported a gradual increase in the acetylation levels of H3K14 during *in vivo* and *in vitro* post-ovulatory aging of murine oocytes. In mice, oocyte ageing induced by advanced maternal age experience downregulation at transcript level of *sirt2*.

Altered intensity acetylation of H4K12 was recorded during *in vitro* post ovulatory ageing of grass carp oocytes. Decreased intensity acetylation of H4K12 was obvious on the oocytes aged as soon as 1 hour post ovulation and continued to decrease until 4 hours post ovulation. This suggests that the mechanism that regulate histone deacetylation might be impaired in aged oocytes during incubation *in vitro*. No significant changes were observed for the H4K12ac up to 8 HPO *in vivo* and 8 HPS *in vitro* oocyte ageing in common carp, that the time oocytes were fertilizable. However, the general trend showed decreasing histone H4K12 acetylation at 8 hours, either *in vivo* or *in vitro* oocyte ageing in common carp (Waghmare et al., 2021b). A significant increase in H4K12ac was observed after losing oocyte fertilising ability at 28 HPS *in vitro* during common carp oocyte ageing (Waghmare et al., 2021b). In addition, the observed decreasing trend for H4K12ac in common carp and grass carp during the time oocytes holding the fertilizing ability seems in line. The observed decrase in H4K12ac is in contrary with the previously obtained results by Huang et al. (2007) and Cui et al. (2011)

who suggested that acetylation levels of H4K12 in murine and porcine oocytes increased significantly during *in vitro* oocyte ageing. An artificial delay in the progress of oocyte ageing would cause a significantly decreased H3 and H4 acetylation (Huang et al. 2007). Additionally, these authors were able to detect increased histone acetylation levels of histones H3 and H4 by accelerating oocyte ageing. Another study reports the signal for the H4K12 acetylation in 12 hours aged mouse oocyte while no signal was detected in freshly ovulated oocytes (Liu et al., 2009). In addition, the increased fluorescence signals for H4K12ac was reported in 24 h-aged mouse oocytes (Trapphoff et al., 2016). Gu et al. (2010) suggested that histone acetylation patterns in oocytes are species-specific. Therefore, different patterns of H4K12 acetylation observed during the progress of oocyte ageing in different studies mentioned above might be attributed to the different organisms studied. RPD3 is one of the factors involved in regulating H4K12 deacetylation status (Rundlett et al., 1996). The up-regulation and down-regulation of the transcripts are also connected with the acetylation or deacetylation status of the histone proteins associated with the respective genes (Delage and Dashwood, 2008). In the future studies it would be therefore of interest to look at the regulation of associated genes during the progress of oocyte ageing.

The underlying pathways of the oocyte ageing effects on histone acetylation are unclear (Gu et al., 2010). Several studies point that oocyte ageing causes a decrease in mitochondrial membrane potential (Wilding et al., 2001). As a result of defective mitochondria function, the level of ATP decreases and the production of ROS increases (Tarin, 1996). Increased ROS and subsequently oxidative stress can modify histone acetylation status in ooplasm (Cui et al., 2011). Cui et al. (2011) reported increased acetylation levels of histone H4K12 during *in vitro* porcine oocyte ageing and suggested it is attributed to increase in ooplasmic ROS. However, our previous studies indicate that oxidative stress is not the main initiator of post-ovulatory ageing process in tench (*Tinca tinca*), common carp and goldfish (*Carassius auratus*) during oocyte ageing (Samarin et al., 2018; Samarin et al., 2019a,b). H4K12ac is required for accurate chromosome segregation during cell division (Régnier et al., 2005). Decreased acetylation levels of histone H4 lysine 12 in the present study might affect the chromosome segregation and lead to chromosomal abnormalities. Histone acetylation modification as an important epigenetic regulator is reported to be related to chromosome segregation (Gu et al., 2010). Therefore, defects in deacetylation at histone H4 lysine 12 in old oocyte may lead to high frequency of aneuploidy. It is now stablished that a decline in fertilization rate as a result of oocyte ageing is related with high frequency of chromosome segregation defects and an accompanying increase in aneuploidy (Hamatani et al., 2004). Ploidy anomaly is a major pathological phenotype of post-ovulatory oocyte ageing in several fish species. Ova ageing in rainbow trout (*Oncorhynchus mykiss*) (Aegerter and Jalabert, 2004), tench (*Tinca tinca*) (Flajšhans et al., 2007), pikeperch (*Sander lucioperca*) (Samarin et al., 2015a), Northern pike (*Esox lucius*) (Samarin et al., 2016) and zebrafish (*Danio rerio*) (Waghmare et al., 2021a) has been shown to be associated with the increased occurrence of triploidization.

Histone acetylases and deacetylases are among the multiple enzymatic systems assuring the generation and maintenance of epigenetic patterns. The balance between acetylation and deacetylation modifications is achieved by the activity of the enzymes histone acetyltransferases (HATs) and histone deacetylases (HDACs). Changes in enzymes including histone acetyltransferases (HATs and MYSTs) and histone deacetylase (HDACs) might be the direct reasons for epigenetic alterations in aged oocytes. In the present study we observed an increasing trend in HAT activity during 1h, 4 h and 30 hours *in vitro* oocyte ageing; however, we observed statistical significance in enzyme activity after 30 hours. In another study we observed an upward trend in the activity of histone acetyltransferase during the progress of oocyte ageing (Waghmare et al., 2021b). In the current study, the histone H4K12ac decreased

significantly while the HAT activity didn't change significantly at 1 HPS and 4 HPS. The increase in HAT activity at 30 HPS and the possible effect on the histone acetylation status during this advanced ageing time needs to be investigated in grass carp. However, our previous study in common carp reported a significant increase in H4K12ac at 28 HPS aged oocytes *in vitro* (Waghmare et al., 2021b). These observations suggest the possible conserved nature of histone acetylation regulation in common carp and grass carp. Similarly, in porcine, an increased level of *hat1* transcript was reported in 24 hours *in vitro* aged oocytes compared to fresh MII oocytes (Cui et al., 2011). In addition, a similar significant surge of *hat1* transcript level was also observed at 48 and 60 hours *in vitro* aged goat oocytes (Zhang et al., 2013). The observed trend of HAT activity during the post-ovulatory oocyte ageing in fish is in line with previous reports in porcine and goat. Therefore, these observations indicate the strong involvement of impaired HAT activity during the progress of oocyte ageing. Further, the observed significant decrease in H4K12ac at 1 and 4 HPS suggests increased deacetylase activity during the oocyte ageing. A similar trend of decreased H4K12ac was observed at 8 hours, either *in vivo* or *in vitro* oocyte ageing in common carp (Waghmare et al., 2021b). However, in mouse, the transcript levels of genes responsible for histone deacetylation (*sirt1*, *sirt2*, and *sirt3*) were downregulated during *in vivo* and *in vitro* oocyte ageing (Jiang et al., 2011). However, there are several histone deacetylases (e.g., HDAC1, HDAC2, HDAC3, SIRT1, SIRT2, SIRT3) that removes the acetyl group from the specific lysines on specific histone proteins (Nicholson et al., 2015). Therefore, it will be noteworthy to investigate the global histone deacetylase activity during the progress of oocyte ageing.

5. Conclusion

Comparison of acetylation status of selected histone lysins between freshly ovulated and post-ovulatory aged oocytes by western blotting revealed stable histone acetylation pattern for most of the modifications examined in this study. Notably decreased acetylation levels of H4K12 was observed in oocytes after 1 hour and 4 hours of *in vitro* post-ovulatory ageing. This suggests that the mechanism that regulate histone deacetylation might be impaired in aged oocytes during incubation *in vitro*. However, reduced viability rates and altered histone H4K12 acetylation indicate that post-ovulatory ageing affects maternal factors, which might lead to a reduced developmental competence. Additional research is needed to explain the relationships between embryo's developmental competence and histone acetylation levels in aged oocytes. Alterations in the expression of histone acetyltransferases (HATs and MYSTs) and histone deacetylase (HDACs) during oocyte ageing can directly affect epigenetic related modifications. Additional research, however, is needed to clarify whether/how ageing can induce changes in their expression.

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CHAPTER 5

General discussion

The ability of the oocyte to become fertilized and develop into the normal embryo declines with increased postovulatory oocyte age, which starts at ovulation and progresses continuously. The resulting decline in oocyte quality leads to major consequences, such as limited fertilization, increased embryo mortality and elevated abnormalities in the progeny. Depending on the fish species and oocyte storage temperature, the fertilization ability of oocytes is maintained for a few minutes to a few weeks after ovulation (Kjørsvik et al., 1990; Samarin et al., 2015). The egg phenotype and functional changes during fish oocyte ageing have been reported in several species (reviewed by Samarin et al., 2015). The current study, intended to indicate the successful egg *in vitro* storage duration in zebrafish (AB strain), which is an important model organism, and in grass carp, which is an economically valuable freshwater farmed species. The fertilization, embryo survival, and hatching rates significantly decreased over the ageing period in zebrafish. In our experiment with zebrafish, the oocytes could keep their fertilizing ability unchanged for up to 2 hours post-stripping at the storage temperature of 26 °C. A study in oocytes from a wild strain of zebrafish showed that the fertilization rate does not change between 1 and 2 hours of storage at 23 °C in Hanks' buffered saline supplemented with 0.5% bovine serum albumin (BSA) (Sakai et al., 1997). Based on fertilization and embryo developmental rates, rapid oocyte ageing was observed in less than 1 hour of *in vitro* storage at 8 °C in golden zebrafish (medium containing Hanks' saline supplemented with 1.5 g of BSA and 0.1 g of sodium chloride) (Cardona-Costa et al., 2009). Furthermore, we observed a significant increase in zebrafish embryo mortality and larval malformation rates during oocyte ageing; all the larvae derived from the 4- and 6-h aged oocytes were characterized by physical abnormalities. Based on our obtained results, we suggest the possible maintenance and manipulation of AB strain zebrafish oocytes at 26 °C for 2 hours after ovulation without a marked reduction in their fertilization potential. In grass carp, the survival rates of the 24-hour embryos remained unchanged up to only 30 minutes poststripping of the oocytes. The values decreased significantly at 1 hour and thereafter remained constant up to 4 hours.

Circadian rhythm is a daily rhythm of physiological, biochemical, and behavioural processes driven by intrinsic circadian oscillators that function in connection with the environmental light-dark cycles. Natural spawning in zebrafish is a noticeable process, with a circadian rhythm that usually occurs within the first minute of exposure to light following darkness (reviewed by Krylov et al., 2021). Alteration in the circadian rhythm during early embryo development of zebrafish has a direct effect on the developmental performance and hatching rates as well as the hatching time (Villamizar et al., 2013). The exposure of zebrafish embryos to the continuous light or dark conditions led to delayed hatching (Villamizar et al., 2013). In the current study, the zebrafish embryos originating from the fresh and aged oocytes didn't experience the same photoperiod conditions during the early stages of embryogenesis. Therefore, the observed decrease in the hatching rates of embryos raised from the more aged oocytes could be partially attributed to the disturbed circadian rhythm due to the ageing. The molecular circadian rhythm network in zebrafish is known (Delaunay et al., 2000; Danilova et al., 2004; Yang et al., 2019). Investigating these regulators and their possible connection to the aged oocytes and the embryos originating from those would be worth to address in the future studies.

The elapsed postovulatory time not only affects fertilizing ability of the oocytes, and the embryo survival rates, but also leads to increasing the possibility of arising abnormal embryos. Chromosome segregation defects and an accompanying increase in ploidy anomalies have been introduced as a major pathological phenotype of postovulatory oocyte ageing in several

fish species (*e.g.*, Aegerter and Jalabert, 2004; Flajšhans et al., 2007; Samarin et al., 2016). An increasing incidence of ploidy anomalies in fish embryos as a result of oocyte ageing has been reported in trout (*Oncorhynchus mykiss*) (Aegerter and Jalabert, 2004), tench (*Tinca tinca*) (Flajšhans et al., 2007), Japanese eel (*Anguilla japonica*) (Nomura et al., 2013), northern pike (*Esox lucius*) (Samarin et al., 2016), and yellowtail tetra (*Astyanax altiparanae*) (do Nascimento et al., 2018). Compared to 0- and 2-hour aged oocytes *in vitro*, the incidence of ploidy anomalies increased significantly at 4 hours post stripping (HPS) in zebrafish. Based on examination of ploidy anomalies in 24-hour embryos and in the newly hatched larvae of zebrafish, we strongly suggest detecting ploidy abnormalities before egg hatching, as the embryos with ploidy anomalies die at this time and become unavailable for the calculations. A close correlation between oocyte ageing and a decrease in adenosine triphosphate (ATP) content was found during oocyte ageing in common carp (Boulekbache et al., 1989) and rainbow trout (Aegerter and Jalabert, 2004). Decreases in ATP content and mitochondrial dysfunction are among the crucial factors that lead to chromosomal abnormalities (Igarashi et al., 2014). The ATP content in aged oocytes suggested the abnormal assembly of the cytoskeleton and spindle contractile proteins, which could lead to an increase in ploidy anomalies in embryos arising from aged oocytes in common carp and mice (Boulekbache et al., 1989; Igarashi et al., 2014). In addition, chromosomal mis-segregation has also been reported as a consequence of abnormal histone acetylation in horse oocytes (Franciosi et al., 2017). Our preliminary results with zebrafish indicated a decrease in the mRNA levels of *mad2* (mitotic arrest deficient protein) transcripts in 4-h aged oocytes and embryos originating from those oocytes (unpublished data). *mad2*, a spindle assembly checkpoint (*sac*), is responsible for genome stability and accurate chromosome segregation. The decreased mRNA levels could be one of the reasons behind the increased ploidy anomalies in zebrafish embryos originating from aged oocytes. Among the various epigenetic regulators, histone acetylation has been reported to be associated with chromosome segregation (reviewed by Gu et al., 2010). A detailed study of the association of fish oocyte ageing and the incidence of ploidy anomalies at the molecular level is of high interest for future works.

The molecular mechanisms driving oocyte ageing is not yet evidently illustrated in fish or in other vertebrates. There are controversies regarding the mechanisms behind the oocyte ageing process and its associations with developing embryo defects and offspring health status. Epigenetic alterations are categorized as primary hallmarks of ageing that are unambiguously negative and are causes of damage (Lopez-Otin et al., 2013). Thus, epigenetic modification in postovulatory aged oocytes may be altered and has been proposed as a likely contribution to the ageing phenotype (Liang et al., 2012; McCauley and Dang, 2014). Among various posttranslational modifications, histone acetylation has been reported to be influenced by postovulatory oocyte ageing in other vertebrates (reviewed by Liang et al., 2012). In the present study dynamics of histone acetylation, as important epigenetic regulators in common carp and grass carp oocytes were investigated. The highly conserved lysines vulnerable to acetylation modification, K9 and K14 on H3 and K5, K8, K12 and K16 on H4, were selected for examination at different aged oocytes. These selected lysine acetylation sites have been described as active transcription marks (Gu et al., 2010; Cunliffe, 2016), and therefore, their alteration could directly influence the normal balance of the developmental potential of oocytes and developing embryos.

In our study, the global histone modifications didn't change significantly during 8 h of oocyte storage in common carp either *in vivo* or *in vitro*. However, at a lower significance level ($p<0.01$), some of the protein spots showed dynamic changes. The acetylation status of histones H3K9, H4K5 and H4K8 did not change significantly during 28 h *in vivo* and *in vitro* oocyte ageing in common carp and 4 h *in vitro* oocyte ageing in grass carp. This observation suggests the

stable and conserved nature of H3K9, H4K5 and H4K8 acetylation marks in common carp and grass carp metaphase II (MII) oocytes. Moreover, as both the studied fish species belong to the same family, Cyprinidae, the mechanisms of regulation of histone modifications might be conserved. In addition, when comparing *in vivo* and *in vitro* oocyte ageing at 8 and 28 HPS in common carp, none of the selected histone acetylation marks showed significant differences. This observation suggests the possibility of similar controlling mechanisms during oocyte ageing *in vivo* or *in vitro* in common carp. A study in mice have reported no signal for H3K9, H4K5 and H4K8 acetylation during *in vivo* and *in vitro* oocyte ageing (Huang et al., 2007). In contrast, another study in mice reported an increase in H3K9 acetylation (Xing et al., 2021). The authors attributed the contrary results to different oocyte culture media and antibodies used in the experiment (Xing et al., 2021). In the current study, no signal was detected for histone H3K14 and H4K16 acetylation marks in fresh or aged oocytes in the common carp and grass carp. Similarly, this observation suggests the conserved nature of histone H3K14 and H4K16 acetylation marks in common carp and grass carp MII oocytes. Huang et al. (2007) reported the gradual increase in H3K14ac in mouse aged oocytes and the complete absence in fresh oocytes. Furthermore, the acetylation of histone H4K16 was not detected during *in vivo* and *in vitro* mouse oocyte ageing (Huang et al., 2007). A signal for acetylation on H3K14 was also not detected in fresh mouse oocytes, but observed in the aged oocytes (Liu et al., 2009). The studied acetylation marks are species-specific and oocyte stage-dependent (reviewed by Gu et al., 2010).

The acetylation status of histone H4K12 increased significantly at 28 HPS during *in vitro* oocyte ageing in common carp. The increase in H4K12ac (28 h) was observed much further after the complete loss of oocyte fertilizing ability (14 h) of in common carp. However, H4K12ac decreased significantly at 1 HPS and continued to decline until 4 HPS in grass carp oocytes. A decrease in H4K12ac in grass carp oocytes was observed within the time period in which oocytes were still fertilizable. Our observations suggest that the mechanisms controlling histone acetylation in oocytes are affected by postovulatory oocyte ageing. At the same time, it can also be realized that the impaired mechanisms regulating histone acetylation lead to a progressive loss in egg quality during postovulatory oocyte ageing in fish. Reports on other vertebrates similarly have indicated impaired histone acetylation during oocyte ageing. H4K12ac was shown to increase significantly during *in vivo* and *in vitro* murine oocyte ageing (Huang et al., 2007). In porcine oocytes, H4K12ac was shown to increase during *in vitro* oocyte ageing and was attributed to an increase in ooplasmic ROS (Cui et al., 2011). However, previous studies have indicated that oxidative stress is not the main initiator of the postovulatory ageing process in common carp, goldfish, and tench during oocyte ageing (Samarin et al., 2018, 2019a,b). Furthermore, H4K12ac is ultimately involved in accurate chromosome segregation during cell division (Régnier et al., 2005). Therefore, the reported increase in ploidy anomalies during fish oocyte ageing could also be attributed to the diminished mechanism of histone acetylation.

The cascades of biochemical processes that occur postfertilization or after egg activation are also initiated by the zygotic clock (Schulz and Harrison, 2019). Zygotic genome activation is a process by which embryonic transcription begins. Posttranslational modifications of histone proteins have been reported to be involved in zygotic genome activation at the correct time (Sato et al., 2019). Furthermore, histone acetylation is proposed to increase during the maternal to zygotic transition and is linked to the activation of genes (Li et al., 2014; Sato et al., 2019). Therefore, the observed H4K12 hyperacetylation in common carp aged oocytes might be due to the spontaneous activation of the zygotic clock. The oocyte ageing may trigger some pathways leading to non-completed zygotic genome activation and partially explain the foremost consequences.

In the current study, the HAT enzyme activity was assessed during oocyte ageing to elaborate the observed specific hypo- or hyperacetylation of histones. During common carp *in vivo* and *in vitro* oocyte ageing, the HAT activity did not change significantly; however, it showed an increasing trend. Furthermore, we observed a significant increase in HAT activity during advanced *in vitro* oocyte ageing (30 HPS) in grass carp. Therefore, analysing the specific histone acetylation as the age-related marks are suggested not only up to the time point of losing oocyte fertilizing ability, but also in the advanced aged oocytes to provide more insights. Histone H4K12ac in grass carp oocytes decreased significantly, while HAT activity did not change significantly during this period. Several histone acetyltransferases (*e.g.*, HAT1, KAT1, KAT2A,) are involved in the acetylation of specific lysines on specific histone proteins and several histone deacetylases (*e.g.*, HDAC1, HDAC2, HDAC3, SIRT1, SIRT2, SIRT3) remove the acetyl group from the specific lysines on specific histone proteins (Nicholson et al., 2015). Therefore, the general contradictory trend of decreasing H4K12ac and increasing HAT activity might be better explained when the activity of individual enzymes involved in the acetylation and deacetylation will be separately examined.

Previous studies in pigs (Cui et al., 2011) and goat (Zhang et al., 2013) reported the increase in histone acetyltransferase *hat1* transcript during oocyte ageing. The transcript levels of genes responsible for histone deacetylation (*sirt1*, *sirt2*, and *sirt3*) were found to be downregulated during *in vivo* and *in vitro* mouse oocyte ageing (Jiang et al., 2011). Unpublished data from our group on zebrafish oocyte ageing demonstrated a significant decrease in the transcription levels of genes involved in histone deacetylation (*hdac9b* and *sirt1*) in embryos arising from aged oocytes compared with those derived from fresh oocytes. Our obtained results, indicated the decrease in H4K12ac in more advanced aged oocytes (28 h) of common carp, and the increase of HAT activity in more advanced aged oocytes (30 h) in grass carp. These findings may suggest the late onset of the histone acetylation regulatory mechanisms in the aged fish oocytes.

In the current study, individual variations were found among the different individuals in studied histone acetylation marks in both common carp and grass carp. When compared these individual variations with the egg viability parameters, a possible negative correlation between H3K9, H4K5, H4K8 and H4K12 hyperacetylation and egg viability parameters were observed. The proteomic investigation in poor quality zebrafish oocytes suggested the deficiency of proteins involved in protein synthesis, energy metabolism and lipid metabolism, and excess of proteins involved in endo-lysosomal activities, including autophagy, apoptosis and oncogenes (Yilmaz et al., 2017). In addition, the transcriptomic analysis revealed the dysregulation of the translational machinery genes in poor quality zebrafish oocytes (Cheung et al., 2019). However, there is limited information about the epigenetic modifications associated with fish egg quality. As the post-fertilization success and development of healthy offspring require the establishment of proper epigenetic marks (Ge et al., 2015; Labbé et al., 2017), it will be noteworthy to investigate epigenetic alteration in gametes as well as the arising progeny of varying quality oocytes.

The classical method, Western blotting, applied in the current study, describes the protein's expression levels. To further investigate the genomic regions associated with specific histone hyper or hypo acetylation during the fish oocyte ageing, advanced methods such as chromatin immunoprecipitation sequencing (ChIP-seq) can be applied. ChIP-seq has previously been employed to investigate the dynamic regulation of H3K4me3 and H3K27me3 modifications at the promoter regions of specific genes in gametes and during early embryo development in zebrafish (Zhu et al., 2019). Beyond, the newest techniques like assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-Seq) helps to screen the accessible chromatin regions (open/closed chromatin structure) and, therefore, would provide

genome-wide information of open chromatin structure. ATAC-seq requires comparatively small numbers of cells. In addition, comparing the results of ATAC-Seq with that of ChIP-seq data would provide more insights into the function of specific histone modifications. Therefore, utilizing such advanced techniques to investigate the epigenetic regulation during oocyte ageing would give a much better portrait and is interesting for future studies.

The possibility of modifying oocyte ageing duration by inducing histone acetylation has been reviewed by Liang et al., 2012. Artificially delayed post-ovulatory ageing by caffeine in mouse oocytes leads to hypoacetylation at lysine residues (Huang et al., 2007). Furthermore, it was demonstrated that artificial acceleration of oocyte ageing progress applying TSA (Trichostatin A) correlates to hyperacetylation at lysine residues (Yoshida et al., 1990; Huang et al., 2007). This evidence indicates that epigenetic modifications might be one of the underlying mechanisms of post-ovulatory oocyte ageing. Such strategies to delay the oocyte ageing in fish could be explored in future. Besides histone acetylation, the other posttranslational modifications, such as methylation and phosphorylation could be equally important to investigate during fish oocyte ageing. As the histone post-translational marks are involved in epigenetic regulation of gene expression, investigating the dynamics of histone modifications in embryos arising from different aged oocytes could be addressed in future studies.

Conclusions

Considerable defects in egg fertilization ability and developmental success are observed during fish oocyte ageing. The present study reports the absence of H3K14ac and H4K16ac and the presence of H3K9ac, H4K5ac, H4K8ac and H4K12ac in metaphase II stage oocytes of common carp and grass carp. Comparison of the acetylation status of selected histone lysines (H3K9, H4K5 and H4K8) between freshly ovulated and aged oocytes of common carp and grass carp revealed a stable histone acetylation pattern for those modifications. During the 4-h *in vitro* ageing of grass carp oocytes, H4K12ac was significantly deacetylated, while in common carp, the same modification increased significantly in 28-h aged oocytes *in vitro*. Hence, posttranslational modification of H4K12ac may be species-specific and contribute to oocyte ageing. The HAT activity increased significantly during advanced oocyte ageing in grass carp. For the future, it is interesting to investigate whether post-ovulatory oocyte ageing induces histone modifications in resulting embryos from different aged oocytes. As histone modifications have been seen as species-specific, employing modern techniques such as ChIP-seq and ATAC-seq in combination would help identify the possible epigenetic markers for oocyte ageing in commercially important fish species. Rapid antibody kits could be developed for such identified epigenetic markers and utilized at commercial hatcheries to boost aquaculture production.

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English summary

Fish oocyte ageing and histone modifications

Swapnil Gorakh Waghmare

Cellular ageing is characterized by a loss of functional abilities over time. Epigenetic changes are one of the factors that contribute to ageing. Epigenetic regulators control cellular gene activity without any alteration of the underlying DNA sequence. Epigenetic regulators such as DNA methylation, histone modifications, and noncoding RNAs have been related to the oocyte ageing process in other vertebrates. Histone modifications are among the most crucial and common epigenetic configurations that play a critical role in postfertilization success and early embryo development. Oocytes carry essential information to orchestrate embryogenesis and to remodel the parental genomes. The fertilization, embryo quality and normal development of offspring are highly related to oocyte integrity. The underlying molecular pathways driving oocyte ageing remain unknown. Investigations and an understanding of the molecular mechanisms behind oocyte ageing are needed. If oocyte ageing affects epigenetic regulators, it is crucial to identify and understand the molecular pathways involved and their regulation.

Therefore, oocyte ageing was investigated, focusing on histone modifications. Egg phenotype and functional changes during fish oocyte ageing were examined. Despite being widely used as a model organism, the impact of oocyte ageing on ploidy abnormalities has not yet been studied in zebrafish (*Danio rerio*). Therefore, an investigation of ploidy anomalies in embryos originating from different aged oocytes in zebrafish was performed. In the molecular analysis, histone modifications, with a special focus on acetylation, were assessed. The key acetylation sites on two lysines on histone H3 (K9 and K14) and four lysines on histone H4 (K5, K8, K12 and K16) were chosen as specific modifications. Histone acetyltransferase activity was assessed to obtain more information on the dynamics of histone acetylation during fish oocyte ageing.

The current study reports that the maintenance and manipulation of zebrafish oocytes at 26 °C for 2 hours poststripping (HPS) was possible without significantly reducing fertilization potential. Almost complete loss of egg viability was observed at 6 HPS in zebrafish. In addition, physical abnormalities were observed in all larvae derived from 4 and 6 HPS-old oocytes. Compared to 0 and 2 HPS, the proportion of embryos with abnormal ploidy was significantly greater at 4 HPS in zebrafish. The egg eyeing and hatching rates were significantly decreased with the increasing time of oocyte ageing in common carp (*Cyprinus carpio*). A significant decrease in the embryo survival rate at 1 HPS was observed in grass carp (*Ctenopharyngodon idella*). The analysis of global histone modifications showed no significant changes for up to 8 hours during *in vivo* and *in vitro* common carp oocyte ageing. The specific histone acetylation of H3K9, H4K5 and H4K8 did not exhibit significant differences in different aged oocytes of common carp and grass carp. However, histone H3K14ac and H4K16ac did not show a signal in either fresh or aged oocytes in common carp and grass carp.

H4K12ac increased significantly at 28 HPS in common carp but decreased significantly at 1 and 4 HPS in grass carp. Histone acetyltransferase (HAT) activity showed an increasing trend during *in vivo* and *in vitro* oocyte ageing in common carp. The HAT activity increased significantly at 30 HPS in grass carp. The observed histone acetylation marks are reported to be species-specific and oocyte stage dependent. Therefore, the obtained results are the first to report the presence of H3K9ac, H4K5ac, H4K8ac, and H4K12ac, as well as the absence of H3K14ac and H4K16ac in common carp and grass carp metaphase II oocytes. Further research into other histone modifications and the associated genomic regions appears to be worthwhile.

Czech summary

Stárnutí rybích oocytů a modifikace histonů

Swapnil Gorakh Waghmare

Buněčné stárnutí oocytů je charakterizováno ztrátou jejich funkčních schopností v průběhu času. Epigenetické změny jsou jedním z faktorů, které přispívají k procesu stárnutí oocytů. Epigenetické regulátory, jako je metylace DNA, modifikace histonů a nekódující RNA, řídí aktivitu buněčného genu bez změny základní sekvence DNA. Tyto regulátory souvisejí s procesem stárnutí oocytů také u jiných vyšších obratlovců. Modifikace histonů patří mezi nejdůležitější a nejběžnější epigenetické změny, které hrají klíčovou roli a významně ovlivňují úspěšnost vývoje zárodku těsně po oplození a při rané embryogenezi. Proces oplození oocytů, kvalita embryí a normální vývoj larev úzce souvisí s integritou oocytů. Základní molekulární procesy a dráhy způsobující stárnutí oocytů zůstávají však stále neznámé. Z tohoto důvodu je zapotřebí se nadále věnovat výzkumu, sledování a porozumění molekulárním mechanizmům, které jsou zodpovědné za proces stárnutím oocytů. Pokud stárnutí oocytů ovlivňuje epigenetické regulátory, je klíčové identifikovat a porozumět zapojeným molekulárním procesům a drahám včetně jejich regulace.

Tématem této dizertační práce bylo sledování procesu stárnutí oocytů u ryb se zaměřením na modifikaci histonů. Vedle toho byl také sledován a zkoumán fenotyp oocytů a jejich funkční změny během stárnutí. V práci byly především využívány oocyty zebřičky pruhované (*Danio rerio*) jako významného modelového organizmu, u kterého prozatím vliv stárnutí oocytů na ploidní abnormality embryí nebyl studován. Především byla ale sledována modifikace histonů se zvláštním zaměřením na acetylaci histonů. Vybrána byla acetylační místa na dvou lyzinech na histonu H3 (K9 a K14) a čtyřech lyzinech na histonu H4. Pro získání podrobnějších informací o dynamice acetylace histonů během stárnutí rybích oocytů byla hodnocena i aktivita histon acetyltransferázy.

Oocyty dánia pruhovaného, které byly uchovány při 26 °C po dobu 2 hodin po jejich umělém výtěru (HPV), vykázaly oplozenost bez výrazného snížení. Téměř úplná ztráta životaschopnosti a oplození schopnosti byla pozorována až po 6 h od umělého výtěru ryb. Kromě toho byly u všech larev získaných z oocytů uchovávaných 4 a 6 h po umělém výtěru pozorovány morfologické abnormality. Ve srovnání s oocyty, které byly uchovávány do 2 h po umělém výtěru, byl podíl ploidních abnormálních embryí zebřičky pruhované významně vyšší u oocytů uchovávaných 4 h po umělém výtěru. Míra přežití embryí do očních bodů a míra líhnivosti se významně snižovala v průběhu postupného stárnutí oocytů i u kapra obecného (*Cyprinus carpio*). U amura bílého (*Ctenopharyngodon idella*) byl pozorován významný pokles míry přežití embryí již u oocytů, které byly uchovávány 1 h po umělém výtěru. Analýza modifikací histonů neprokázala žádné významné změny po *in vivo* a *in vitro* uchování oocytů kapra obecného po dobu do 8 h od výtěru. Specifická acetylace histonů na H3K9, H4K5 a H4K8 nevykazovala významné rozdíly u různě starých oocytů kapra obecného a amura bílého. Histony H3K14ac a H4K16ac nevykázaly žádnou zásadní změnu u čerstvých ani u starších oocytů kapra obecného a amura bílého. Histon H4K12ac se začal ve významné míře objevovat u oocytů kapra obecného uchovávaných 28 h po umělém výtěru, u oocytů amura bílého byl zanedbatelný v časech 1 a 4 h po umělém výtěru. Aktivita histon acetyltransferázy (HAT) vykazovala rostoucí trend během procesu stárnutí u *in vivo* a *in vitro* držených oocytů kapra obecného. Aktivita HAT se u oocytů amura bílého výrazně zvýšila po 30 h od umělého výtěru.

Získané výsledky týkající se acetylace histonu mají druhově specifický charakter a jsou závislé na stavu oocytu. Jedná se o první výsledky, které popisují přítomnost H3K9ac, H4K5ac, H4K8ac

a H4K12ac, stejně jako nepřítomnost H3K14ac a H4K16ac v metafázi II oocytů kapra obecného a amura bílého. Další výzkum zabývající se modifikací histonů a souvisejících procesů u různě uchovávaných a starých oocytů bude velmi užitečný pro další vysvětlení zákonitostí spojených s procesem stárnutím oocytů.

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List of publications

Peer-reviewed journals with IF

Waghmare, S.G., Samarin, A.M., Samarin, A.M., Danielsen, M., Møller, H.S., Policar, T., Linhart, O., Dalsgaard, T.K., 2021. Histone acetylation dynamics during *in vivo* and *in vitro* oocyte aging in common carp *Cyprinus carpio*. International Journal of Molecular Sciences 22, 6036. https://doi.org/10.3390/ijms22116036. (IF 2019 = 4.556)

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Peer-reviewed journals without IF

Waghmare, S.G., Sindhumole, P., Mathew, D., Shylaja, M.R., Francies, R.M., Abida, P.S., Narayanan Kutty, M.C., 2021. Identification of QTL linked to heat tolerance in rice (*Oryza sativa* L.) using SSR markers through bulked segregant analysis. Electronic Journal of Plant Breeding 12, 46–53. https://doi.org/10.37992/2021.1201.007

Waghmare, S.G., Sindhumole, P., Shylaja, M.R., Mathew, D., Francies, R.M., Abida, P.S. and Sajini, S., 2018. Analysis of simple sequence repeat (SSR) polymorphism between N22 and Uma rice varieties for marker-assisted selection. Electronic Journal of Plant Breeding 9, 511–517.

Salam, S. A., Sindhumole, P., **Waghmare, S.G.,** Sajini, S., 2017. Molecular characterisation of rice (*Oryza sativa* L.) genotypes for drought tolerance using two SSR markers. Electronic Journal of Plant Breeding 8, 474–479.

Abstracts and conference proceedings

Waghmare, S.G., Samarin, A.M., Samarin, A.M., Nguyen, T.V., Franek, R., Pšenička, M., Policar, T., Bobe, J., 2019. Changes in mRNA abundance of selected transcripts during oocyte ageing in zebrafish *Danio rerio*. 7th International Workshop on the Biology of Fish Gametes, Rennes, France, 2–6 September 2019. (Poster presentation)

Barate, P.L., Kumar, R.R., **Waghmare, S.G.,** Pawar, K.R., Tabe, R.H., 2017. Effect of different parameters on Agrobacterium-mediated transformation in Glycine max. EMBO conference on Micro and metabolic regulators in plants. Thiruvananthapuram, India 1–4 February 2017. (Poster presentation)

Rahi, D., **Waghmare, S.G.,** 2017. Phytochemical gene tagging in *Dendrobium officinale* using GFP. EMBO conference on "Micro and metabolic regulators in plants. Thiruvananthapuram, India 1–4 February 2017. (Poster presentation)

Waghmare, S.G., 2016. Mysterious Cell in our Social Life. International conference NextGen Genomics, Biology, Bioinformatics and Technologies, Cochin, India 3–5 October 2016 (Poster and oral presentation)

Training and supervision plan during study

Name	Swapnil Gorakh Waghmare
Research department	2018–2022 – Laboratory of Intensive Aquaculture FFPW
Supervisor	Azin Mohagheghi Samarin, Ph.D.
Period	17th January 2018 until 30th March 2022

Ph.D. courses	**Year**
Pond aquaculture	2018
Basic of scientific communication	2018
Applied hydrobiology	2018
Ichthyology and fish taxonomy	2019
English language	2019

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2019 2020 2021 2022

International conferences	**Year**
Swapnil Gorakh Waghmare, Azin Mohagheghi Samarin, Azadeh Mohagheghi Samarin, Thao Vi Nguyen, Roman Franek, Martin Psenicka, Tomas Policar, Julien Bobe. Changes in mRNA abundance of selected transcripts during oocyte ageing in zebrafish *Danio rerio*. 7th International Workshop on the Biology of Fish Gametes, Rennes, France, September 3–6, 2019. (Poster presentation)	2019

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Research stay, French National Institute for Agricultural Research, INRA, Fish Physiology and Genomics Institute (LPGP), Rennes, France (2 months)	2018
Research stay, Department of Food Science, Aarhus University, Denmark (6 months)	2019
Research stay, Department of Food Science, Aarhus University, Denmark (5 months)	2020–2021

Curriculum vitae

PERSONAL INFORMATION

Name: Swapnil Gorakh
Surname: Waghmare
Title: M.Sc.
Born: 16th April, 1993, Walchandnagar, India
Nationality: Indian
Languages: English, Hindi, Marathi, Czech
Contact: swaghmare@frov.jcu.cz

EDUCATION

2018 – present Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic

2015–2017 M.Sc., Kerala Agricultural University, Thrissur, Kerala, India

2011–2015 B.Sc., VSBT College of Agricultural Biotechnology, Baramati, Mahatma Phule Krishi Vidyapeeth, Maharashtra, India

SCIENTIFIC ACTIVITY AND DEVELOPMENT PROJECTS

2020 Responsible leader of the GAJU project (046/2020/Z) Grant Agency of the University of South Bohemia, Czech Republic

2020–2022 Research member of the Czech Science Foundation project (No. 20-01251S): Epigenetic modifications and functional properties during fish gamete aging

LANGUAGES English IELTS 6.0 Academic, Czech basic level, Hindi fluently, Marathi Native fluency