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DIPLOMA THESIS

“Variability of lipid classes and fatty acid composition during over ripening of oocytes from tench (*Tinca tinca*)”

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Diploma thesis assignment

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1. Introduction and literature overview

Aquaculture is becoming one of the most economically important branch of food services, with an increase in production of animal products of about 9.3% per year since 1985 (Diana 1993, FAO 2005) and exceeding the number of 73.8 million tonnes in year 2014 (FAO 2016). With human population constantly growing, the demand for the fish will also intensify in the future. Fish is a widespread, affordable and healthy source of food. In some cultures it is closely related to tradition and therefore consumed on a daily basis. Both aquaculture and capture fisheries have caused much public concern about their sustainability and influence on the environment (Goldburg and Triplett 1997). If the growing aquaculture industry is to sustain its contribution to world fish supplies, it must reduce wild fish inputs in feed and adopt more improved management practices. To reach that goal successful advanced breeding strategy for aquaculture species is necessary. With a big variety of cultured fish it is important to recognize an individual reproduction approach for each species. Therefore the profound research on fish reproduction is essential to understand and include in fish production management.

Successful reproduction and uninterrupted development of fish is dependent on high quality gametes. Female gamete - ovulated oocyte should be capable of undergoing fertilization and embryonic development that will become a healthy adult fish with no malformation during the larvae stage and in the future. In case of fish, delayed spawning in nature, delayed egg collection in captivity and delayed fertilization after egg stripping can lead to oocyte ageing and over-ripening. Oocyte ageing is reported as one of the most important factors affecting the egg quality in fish (e.g.,Lam*et al,*. 1978; Rime *et al.,* 2004). Decreased fertilization, eyeing, hatching rates and increases in morphological abnormalities or in the appearance of various ploidy abnormalities in larvae are highly connected with oocyte quality decrease (Sakai *et al.,* 1975; Craik and Harvey, 1984; Lahnsteiner, 2000; Aegerter & Jalabert, 2004).

In order to counteract over-ripening and secure successful reproduction of fish it is therefore important to study among others the factors that affect over-ripening and what happens in the oocyte during over-ripening like oxidative stress, zona hardening, cortical granule exocytosis, a decrease in critical cell cycle factors and spindle and chromosomal abnormalities (Samarin *et al.,* 2015).

Fish is a good animal to evaluate oocyte ageing because they represent the big difference in reproductive strategies between species as well as many species produce a high number of oocytes compared with other animals. Studies have been performed on chosen fish species regarding the optimum and maximum time period after ovulation that oocyte remain capable to undergo full fertilization process with no interruptions. The time when over-ripening is reached is highly dependent of fish species and the storage temperature of eggs. It can reach from few minutes to few days in some cases. In case of *Tinca tinca* the precise time of oocyte being able to undergo full fertilization after ovulation is still not well described. Although according to Flaishans *et al.* (2007) after 5 hours in vitro storage of tench eggs should show around 30 % of fertilization rate.

Tench *(Tinca tinca L. 1758)* is a fish species from *cyprindae* family that lives in fresh- or brackish- waters of Central and Southern Europe (Rodríguez *et al*., 2008). It is endemic in this territory and has been traditionally cultured there as well as in Eurasia. Low intensity culture method was mostly used in past. Nowadays with a well developed aquaculture industry and a fast growth of new technologies we are able to produce tench in intensive culture conditions. The optimum parameters are still under investigation as the demand for this *Cyprinid* fish is growing in Czech Republic, Hungary, Italy, Spain and Poland (Kucharczyk *et al*., 2014). According to Mamcarz and Skrzypczak, (2006) and Celada *et al*. (2009) tench has become one of the most promising aquaculture species of freshwater aquaculture in case of lake exploitation and pond breeding. The IUCN, International Union for Conservation of Nature- environmental network status of tench is low (http://www.iucn.org/). According to this, tench is in the list of least concerned species.

A fast growing popularity of this fish however does not mean high success of numbers when it comes to reproduction. The hatching rate of *Tinca tinca* investigated in several studies is very low, usually not higher than 30 % (e.g., Kokurewicz, 1981; Linhart and Kvasnicka, 1992; Flajshans *et al*., 2007). Large earthen ponds with natural vegetation are the place where natural reproduction takes place for tench. Artificial reproduction is applied by using synthetic analogues of gonadotropin releasing factors (GnRH) that are used to induce ovulation (Kouril et al., 1986; Fernández San Juan, 1995; Linhart *et al.,* 1995). Unfortunately data about successful reproduction of wild cyprinid fish under hatchery conditions is limited due to collection of fish

for breeding from natural environment, therefore the number of spawners is always limited (Kujawa *et al*., 2006). Tench is batch spawner and body weigh index (BWI) and size or percentage of the largest oocytes are the applied indexes to identify the suitability of females for artificial reproduction(Pedersen *et al*., 2003; Kucharczyk *et al.,* 2007). These kinds of observations are important for obtaining the best quality eggs

Mass production of fishes requires high quality eggs with good broodstock management that is capable to evaluate the best time for fertilization for specific fish spices especially those that are not hormonally stimulated. Verification of the egg quality and oocyte viability in post ovulatory stage becomes very important in modern aquaculture. Oocyte ageing, both post-ovulatory and post-striping have been associated with limited fertilization rate (Samarin *et al.,* 2015). Aged oocytes can also cause many malformations in embryonic development of fish leading to significant losses in hatcheries.

The oocyte that is in post ovulatory stage is constantly changing. Morphological investigations were followed by biochemical, physiological, and endocrinological analyses that broadened our knowledge on histological, cellular, and molecular level of the dynamic events that take place during oocyte development and egg formation. During prolonged storage of oocytes in vitro for example decrease of egg viability which subsequently leads to over-ripening phenomenon is well documented. In mammalian species oxidative stress has been related to oocyte aging (Lord and Aitken, 2013). However this topic is poorly evaluated in fish and only few studies investigated the lipid changes during oocyte over-ripening and the possible correlation with egg fertilizing ability (Lahnsteiner, 2000). In addition it has not been confirmed if the oocyte ageing is controlled by some specific factor that initiate the series of events that lead to total loss of fertility ability or separate series of events occur that together lead to over-ripening phenomenom (Samarin *et al.,* 2015). Figure 1 shows morphology of egg during process of over-ripening in rainbow trout, showing histological and ultrastructural changes through oocyte ageing. Clearly visible in egg after activation is how cortical vesicles are completely secreted into the perivitelline space (Figure 1b), which is increasing its thickness. Subsequently, pressure on chorion is increasing, leading to decrease in its thickness. In comparison the over-ripened eggs of rainbow trout did not show decrease in chorion thickness due to incomplete extrusion of cortical vesicles (Figure 1e) (Lanhsteiner, 2000).

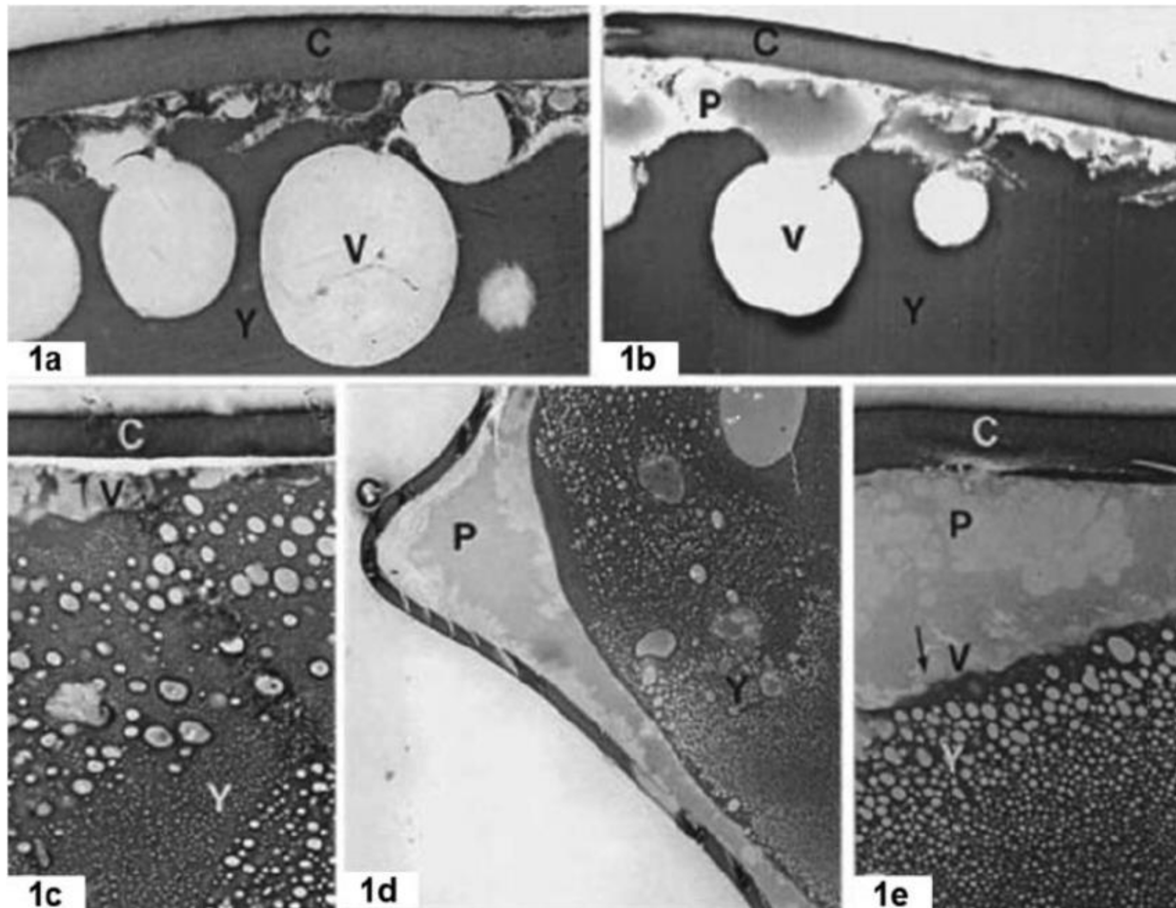

Figure 1. Morphology of eggs of the rainbow trout during over-ripening.

C—chorion; P—perivitelline space; V—cortical vesicle, Y—yolk. (A) Freshly ovulated egg with cortical vesicles before incubation in water. Magnification: 225 x . (B) Freshly ovulated egg during extrusion of cortical vesicles, 5 min after immersion of eggs into water. Magnification: 150 x . (C) Over-ripened egg before incubation in water. Cortical vesicles are located at the plasma membrane. Magnification: 225 x . (D) Over-ripened egg, before incubation in water. Note: enlargement of the perivitelline space and the non homogenous composition of yolk. Magnification: 65 x . (E) Over-ripened egg. Extrusion of cortical vesicles (arrowhead), 5 min after immersion in water. Magnification: 130 x (obtained from Lahnsteiner, 2000).

Nowadays determination of egg quality is done using several parameters (Kjørsvik et .Ž. al., 1990; Bromage and Roberts, 1994). In *Salmonidae* for unfertilized eggs, the transparency and the homogenous distribution of lipid droplets are important criteria of viability description (Craik and Harvey, 1984; Lahnsteiner et al., 1999a). For the fertilized eggs blastomere morphology is a

predictor for the quality as its structure correlates with the viability in several species for example the wolfish *Anarhichas lupus*, the cod *Gadu smorhua* and the Atlantic halibut *Hippoglossus hippoglossus* (Pavlov and Moksness, 1994). In cod the egg viability is affected by intensity of the cortical reaction, egg osmolality and mechanical strength of the chorion (Kjørsvik and Lønning, 1983). Position of the germinal vesicle is the most important factor in evaluating the best time of spawning in *cyprinidae* family (e.g. Brzuska, 1979). However it does not apply to tench as this fish is batch spawner.

The possible use of the analyses of ovarian fluid parameters, egg weight and weight increase during water hardening, biochemical egg composition and egg enzyme activities for prediction of egg quality during short-term storage was investigated in common carp *Cyprinus carpio* and grass carp *Ctenopharyngodon idella*: during short-term storage for 4 h at 48 °C (Lahnsteiner. 2001). However in this study the investigated biochemical parameters of the eggs protein, peptides, fructose, galactose, glucose, non-esterified fatty acids, esterified fatty acids, total DNA and RNA were not correlated with the fertilization rate.

Fatty acid (FA) composition of fish eggs has been evaluated in relation to fertility and significant differences between eggs with different fertility were found. This suggests that FA composition might play a role in over-ripening of oocytes. Increased oxidative stress in aged oocytes has been related with negative effects on mitochondrial function (Takahashi et al. 2013). In mitochondria especially the phospholipid (PL) groups, cardiolipin and sphingomyelin are important for membrane functionality. Both cardiolipin and sphingomyelin are especially sensitive to oxidation due to their high content of polyunsaturated FA, (Paradies *et al*., 2009) and the authors suggested that any change in cardiolipin structure would result in mitochondrial dysfunction. This could also play a role in oocyte aging but further studies are necessary to understand and confirm this mechanism in fish eggs while undergoing oocyte ageing process.

Oxidation is among others majorly caused by radicals. A free radical is any species capable of independent existence that contains one or more unpaired electrons. Over the last four decades, peroxidation of FA induced by free radicals has been studied in great detail as a deleterious process that occurs in plasma as well as in intracellular membranes. The lipid peroxidation reaction is divided into three successive phases: initiation, propagation and termination. Initiation

takes place through an abstraction of a hydrogen atom from a FA containing two or more separated doubled bounds, leading to a carbon-centred alkyl radical, with a simultaneous rearrangement of the double bounds to become conjugated. Thereafter, the alkyl radical formed reacts with oxygen, which is nonpolar and, thereby, soluble in the hydrocarbon core of lipid bilayers, giving rise to a peroxyl radical. Propagation, which involves the abstraction of hydrogen from a neighbouring FA by peroxyl radicals, results in the formation of a lipid hydroperoxide and a new alkyl radical. Finally, termination of the lipid peroxidation process is generally believed to take place by an interaction between two free radicals, resulting in the termination of a nonradical product.

It was for example shown that increased ROS (reactive oxygen species) levels of aged oocytes from species significantly disturb Ca^{2+} homeostasis and mitochondrial function (Takahasi et al. 2003) that can subsequently contribute to apoptosis. Most authors agree with association of oocyte ageing and increase level of ROS. (e.g., Lord et al. 2013). Takahasi already proved that lipid peroxidation in the membranes of in vivo aged oocytes is higher than on fresh ones in mouse model.

In case of tench there are no recent studies performed concerning the lipid composition and content in oocyte while undergoing ageing process. While oocyte ageing has been observed to have connection with oxidative stress (Samarin *et al.,* 2015) we still know very little about changes in lipid structure and composition while ageing in fish species.

FA in general are a group of carboxylic acids that usually contain aliphatic chain which can be saturated or none saturated. That means having double bond, with all of the remainder carbon atoms being single-bonded inside the chain. Unsaturated FA are divided according to the number of double bounds they contain- from monounsaturated with one double bond to polyunsaturated. Those bounds can shape in form of *cis* or *trans* which does affect its physical properties. (Mraz *et al.,* 2009)

Long-chain poly unsaturated FA (PUFA) have more than one double bond inside their chain. Long chain PUFAs are synthesized from essential FA - linoleic acid of omega 6 (n-6) series and α -linolenic acid of the omega 3 (n-3) series (Sprecher, 1999). PUFAs have a general role in

phospholipids (PL) of membrane bilayers to help maintain the structural and functional integrity of the cell (Rojbek *et al*., 2013).

The predominant long chain omega 6 PUFA is arachidonic acid, later converted into prostaglandins, leukotrienes and other lipoxygenase or cyclooxygenase products, vital for cellular function regulations. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) omega-3 FA, are competitive substrates for enzymes and products of arachidonic acid metabolism. In most mammals after obtaining those main FA through diet, they are metabolized within the cells (Marszalek *et al*., 2005). Linoleic acid (18:2 n-6, (LA)) is converted into gamma linolenic acid (18:3 n-6), and dihomo-gamma-linolenic acid (20:3 n-6) to form the important in cell function arachidonic acid (20:4 n-6, (AA)) by various desaturase- and elongase- enzymes. Later AA is converted into docosapentaenoic acid (22:5 n-6) or eicosanoids. The conversion of n-3 FA starts with alfa-linolenic acid (18:3 n-3, (ALA)) being metabolized to stearidonic acid (18:4 n-3) and eicosatetraeonic acid (20:4 n-3) to form EPA (20:5 n-3) by using the same series of enzymes that were involved in AA synthesis. EPA is later modified to become DHA (22:6 n-3) or eicosanoids (Schmitz 2007). It is important to notice that omega-3 and omega-6 fatty acids metabolism track is sharing the same enzymes (Fig. 2) therefore the competition exist between each other with excess of one causing significant decrease in the conversion of the other.

Omega-3 PUFA including EPA, ALA and DHA which play important role in fish as well as in human health. Over the past 20 years, there has been a observe increase in the scientific and public interest in omega-3 and omega- 6 FA and their impact on personal health. Omega-3 FA possess anti-inflammatory, antiarrhythmic, and anti-thrombotic properties while omega-6 FA are pro-inflammatory and pro-thrombotic and can contribute to everything from heart disease to joint pain to skin problems. Fish and fish oil are rich sources of omega- 3 FA, specifically EPA and DHA, which are present in fatty fish and algae. ALA is an omega-3 FA present in nuts and certain plants oils (for example rapeseed and linseed). As well as green leafy vegetables, nuts and beans which are good source of those FA in vegetarian diet. High percentages ofomega-6 FA can be found in vegetable oils such as corn, sunflower, safflower and cottonseed oils. Also meat of animals that were fed feed with high content of omega-6 FA (Covington 2004).

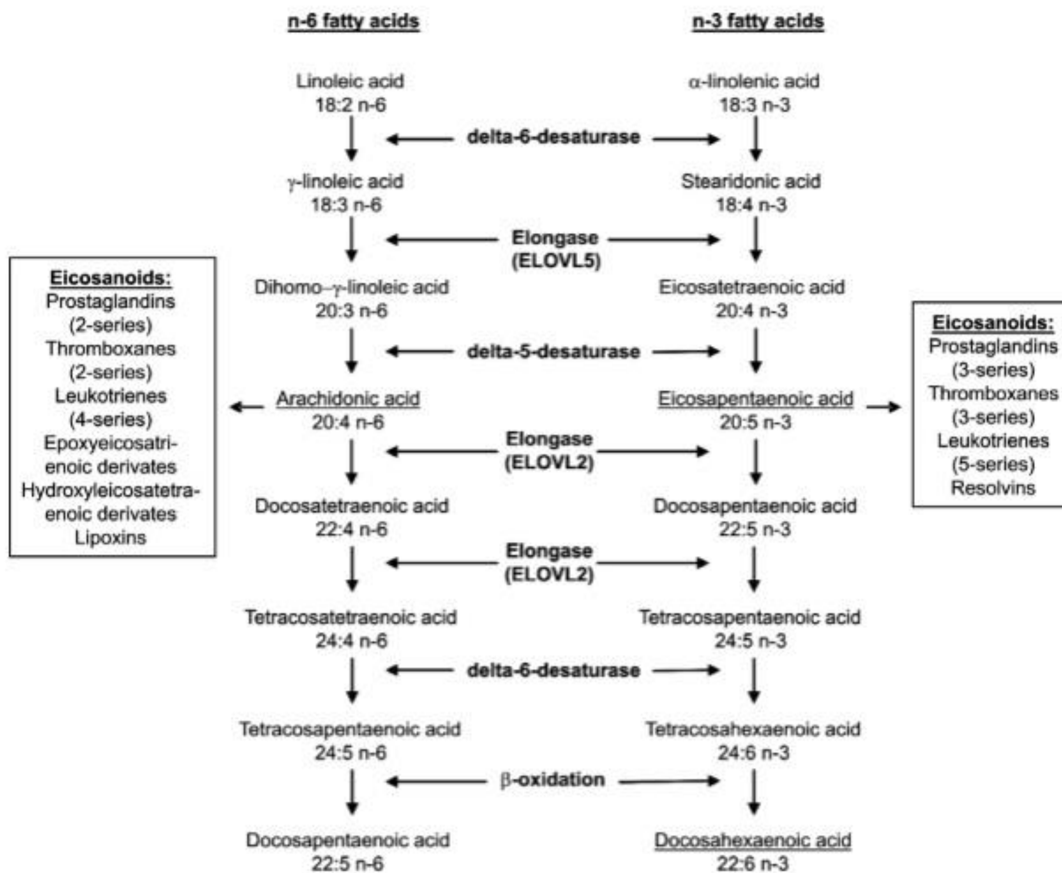

Figure 2. Omega 3 and omega 6 fatty acid metabolism. Obtained from (Wiegand, 1996)

In human, since both types of FA compete to the same enzymes in syntheses, it is known that a healthy omega 6: omega 3 ratio is needed to promote cardiovascular health and to avoid the induction of inflammatory processes. The current recommended omega 6: omega 3 ratio is between 2:1 to 4:1 (Covington, 2004).

Several studies performed on freshwater and salmonid species showed high inclusion levels of plant oil without negative effect on growth an general physiological processes of the fish (Bell *et al.*, 2002. Francis *et al.,* 2006). It has to be considered however, that marine fish differ in FA requirements from those one that live in freshwater. This is mostly due to low ability of marine fish to synthesize highly unsaturated FA (HUFAs) (FA with 20 or more carbon atoms and 5 or more double bonds). Freshwater fish in comparison, are able to synthesize EPA and DHA from ALA as well as AA from LA in sufficient amounts. However, present studies that evaluate

benefits, which can be obtained from dietary HUFA inclusion on freshwater broodstock reproduction performance, are still under investigation as very little is known about HUFA biosynthesis in these freshwater species- especially during the reproductive phase (Jaya-Ram *et al.,* 2008).

Lipids have been considered to be among most important sources of energy in fish eggs, especially in the most common form of triacylglycerol (TAG). In teleost fish lipid reserves are stored in lipoprotein yolk, or discrete oil globule in some species. The content and composition of lipid in fish eggs vary between species and may change during diverse developmental stages according to energy demands of the egg and physiological events that occur inside (Rainuzzo *et al.,* 1997).

In oocytes primarily TAG which differ by species of specific FA are stored in distinct droplet organelles that re-localize during oocyte maturation. The presence of lipids, particularly saturated vs unsaturated FA, in in vitro maturation systems affects oocyte lipid content as well as developmental competence (Dunning, 2014). Lipoprotein yolk lipids are primarily PL phospholipids, especially phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and are rich in omega 3 PUFA like DHA. According to Kaitarana and Ackman (1994) the amount of lipids in fish eggs is correlated to the time interval between spawning and hatching of the egg. They are derived from dietary FA, namely FA mobilized from reserves and possibly FA synthesized *de novo*. During this process FA are incorporated selectively, with a strong pressure on DHA. Lipids in female fish are as well essential in gonadal development as important material of cell and tissue membranes (Sargent, 1995).

Vitollegenin, the precursor protein provides delivery of lipids to the oocyte, rich in PL, mostly lipoproteins that have low density, also in TAG. During embryonic and larval development all classes of lipids can be used in catabolic reactions as fuel with a preference of monounsaturated fatty acids (MUFA). Oil globules are catabolized in the later stages of embryonic development. Some species might also use DHA from hydrolysis of PL by transfer to TAG (Wiegand, 1996).

The HUFAs, a subset of PUFA have been described to be critical for maintaining high growth, good rates of survival and reproduction as well as high food conversion efficiencies for most both

freshwater and marine organisms (Brett, 1997). In case of fish reproduction many studies showed a positive influence of dietary unsaturated omega-3 FA on reproduction rates (Fruita *et al* 2002.) Investigated correlation between feeding of broodstock females during gonadogenesis and qualitative and quantitative lipid contend of feed showed a big impact of those in terms of spawning performance as well as egg quality (Watanebe and Kiron, 1995, Harel *et al.* 1994). Most of the studies performed in this area included marine fish like sea bass (*Dicentrarchus labrax)* (Bell et al. 1996), cod (*Gadus morhua*) (Pickova et al. 1997), gilthead seabream (*Sparus aurata*) (Almansa et al. 1998), greater amberjack broodstock *(Seriola dumerili)* (Rodriguez-Brateto 2014) and anadromous fish like Salmon (*Salmo salar*) (Pickova *et al.,* 1998). Regarding studies performed on cod, two populations were compared to each other (Baltic and Atlantic) in terms of FA composition and successful hatching of their eggs that show significant difference. However in those studies the dietary regime affected PL composition of the yolk only in minor degree. But in similar studies performed on salmon from Swedish rivers it was suggested that the water temperature during oogenesis influences strongly the egg lipid composition by affecting DHA incorporation in eggs resulting in different pattern for each salmon stock. Those studies mostly focus on DHA, EPA, and AA as essential FA for most marine teleost (Sargent *et al.* 2002). All authors agree that supplying proper level of those FA in the broodstock diet is vital for achieving eggs with high quality and content of FA that ensure good embryo and larvae development.

HUFA are also precursors for eicosanoids (prostaglandins, leucotrienes and thromboxanes), very important dynamic molecules that are responsible for cellular activities including gene regulation, signalling and maintenance of cell membrane integrity. Eicosanoids derived from AA are responsible for pheromone attraction, steroidogenesis and oocyte maturation (Henrotte et al 2011). Therefore, the proper amount of essential FA in broodstock diet is necessary and important not only in terms of vitellum composition, but as well as because of its very important role in the regulation of reproductive physiology (Izquierdo *et al*., 2001). Also eicosanoids produced from AA are generally more active than those ones produced from EPA. Both of those products interfere and compete for the same membrane receptors (Sargent *et al*., 1999). That is an explanation for determination of eicosanoids actions by the ratio EPA:AA in cellular membranes which in turn is determined by dietary intake of PUFA.

In fish, ratio of n-3/n-6 is also of great importance, especially in case of reproduction. Jaya-Ram (2008) proved that in zebrafish possible reason for the detrimental effects on reproduction is caused by excess of HUFA in dietary intake and imbalance ratio of FA. Significantly higher n-3/n-6 ratio found in zebra fish ovaries might negatively affect maturation and ovulation. Moreover studies performed on Arctic char*(Salvelinus alpinus)* showed poor hatching rates in farmed fish as compared to wild populations. Those have been linked to higher n-3/n-6 ratio in the farmed fish (Pickova et al., 2007). Also proper ratio of EPA to AA in zebra fish ovaries has been taken into account and shown that a lower ratio could also be connected with impaired reproduction. Several studies focused on this topic as well, showed importance of EPA to AA ratio as important factor in optimal reproduction process (Bell and Sargent, 2003).

The importance of AA in fish is not so well understood yet. Studies performed on cod (*Gadus morhua)* showed significant correlation between feed lipid composition and liver lipid percentages of DHA, EPA and total n-6 FA (Jobling and Leknes, 2010). As well the wild groups, with higher contents of AA showed more success in hatching rates compared to farmed fish with lower AA (Pickova et al. 1999), indicating the importance of this FA for successful reproduction of fish. Importance of AA on breeding rates was also confirmed in studies performed on Japanese flounder (*Paralichthys olivaceus)* where total egg production, percentage of buoyant eggs, hatching success and larval survival were higher when dietary AA intake was increased from 0.6% to 3.6% of total FA. Similar reproductive performance was enhanced in Atlantic halibut *(Hippoglossus hippolossus)* by supplemental dietary AA was added and improve fertilization success, blastomeric morphology score and hatching success (Mazorra *et a*l., 2003).

PL have an important structural function as a main component of double layer cell membrane. As mentioned before, in mitochondria especially cardiolipin and sphingomyelin are important lipids classes for membrane functionality (Paradies *et al.,* 2009).In addition it has been found that cardiolipin is required for an optimal activity of a number of anion carrier proteins and enzymes. Increased ROS in bovine mitochondria resulted in peroxidation of cardiolipin and in addition a distinct loss in cytochrome C oxidase (Paradies *et al*., 2009).Changes in mitochondrial cardiolipin have been found in various tumor cells in mice brain (Kiebish *et al*., 2008). The authors suggest further, that these changes could be due to a variation of causes as FA metabolism, ROS and FA

transport. These aspects could also play a role in oocyte aging. There for our aim is to evaluate the mechanisms of oxidation as well as the possible changes in the lipid composition due to aging or oxidative stress in oocytes.

In rainbow trout over-ripening resulted in increased free lipid and decrease in lipid phosphorus (Craik and Harvey, 1984). Also the levels of esterified and non-esterified FA were significantly decreased in over-ripened rainbow trout eggs (Lahnsteiner, 2000). In Caspian brown trout, the level of triglycerides decreased 30 days post ovulation (Bahrekazemi *et al.,* 2010). In opposite, in vitro storage of common carp and grass carp eggs for 4 hours did not reveal a correlation between the content of non-esterified or esterified FA and the fertilization rates (Lahnsteiner *et al.,* 2001). However these authors only compared the crude fat content and did not evaluate the FA composition or the more specific lipid classes. Therefore present study aimed to investigate the lipid contentand lipid class composition as well as FA composition in oocyte at different ageing stages in tench *Tinca tinca.* This study should add valuable information in aspect of research and more important practical application in aquaculture to prevent or delay the oocyte ageing phenomenon in mentioned fish species. As well as it can be quite beneficial for higher vertebrates studies.

We investigated the lipid content and composition as well as FA composition in oocyte stripped ova of 6 females collected separately and stored in sterile cell culture plates in an incubator at 20 °C for up to 24 hours post stripping (HPS).

2. Material and methods.

2.1 Fish

The broodstock fish were obtain from experimental facilities at South Bohemia Research Centre of Aquaculture and Biodiversity, Hydrocenoses in Vodnany, Czech Republic. In the middle of June 2015, fish were captured from earthen ponds and transferred to indoor facility with rectangular-shaped tanks (each $5.7 m^3$ capacity) supplied with water from a recirculating system and kept under dim conditions (<20 lx). Females and males were kept separately. The water temperature was gradually increased to 21℃. The fish were kept under these conditions for 2 days for acclimation. After this time female brood fish were treated with muscular injections of ovopel (mammalian GnRH analog [D-Ala^6, Pro^9Net-mGnRH] with metoclopramide-dopamine antagonist). First injection dosage was 0.1 and the second one, after 12 h interval, of 1 [pellet kg^{-1}] of female's body weight, (Kujawa *et al.,* 2011). Male brood fish were subjected to a single intramuscular injection of carp pituitary hormone (CPH) (1 mg kg^{-1}) (Linhart et al., 2006) that was injected in the same time as the first hormonal injection of females. CPH was provided by Genetic Fisheries Centre, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic.

After 12 hours of the second injection, females were examined for ovulation every 2 hours. Gentle palpation of the abdomen was used as an evaluation technique of ovulation occurrence. Fish was considered to have already ovulated if after applying gentle pressure to the abdomen eggs could be removed. The inspections were performed every 2 hours thereafter. For this experiment six females which ovulated within 2 hours were randomly chosen. The weight of the female fish was between 0.4 and 1 kg. During the ovulation examination and collection of gametes, fish were anaesthetized with 0.6 ml l^{-1} 2-phenoxyethanol to minimize stress and make handling easier (Flajshans *et al.,* 2007).

2.2 In vitro egg storage in ovarian fluid

Striped ova of 6 females were collected separately and stored in six-well sterile cell culture plates (each well diameter: 3.5 cm). The eggs were stored in two layers (Komrakova and Holtz, 2009).

The eggs were kept in ovarian fluid without any addition of solution, artificial media or extender. All plates were covered by their own lids and stored in dark at the temperature of 20 °C in the laboratory incubator for up to 24 hours. Few plates filled with water were placed into storage chambers to provide a humidified atmosphere (Babiak and Dabrowski, 2003; Komrakova and Holtz, 2009). Stored ova were fertilized at 0 (immediately after stripping), 2, 4, 6, 8, 10 and 24 hours post stripping (HPS).

2.3 Artificial fertilization

The mature males of tench weighing between o.3 and 0.5 kg were randomly selected for the experiment. The milt from each male was collected separately into tubes with modified Kurokura immobilizing solution 180 (180 mMNaCl, 2_68 mMKCl, 1_36 mM $CaCl_2$ $2H_2O$ and 2_38 mM $NaHCO_3$; Rodina *et al.,* 2004) and pooled prior fertilization/further use. 0.5 ml of milt was collected from each of three male for each fertilization step to uniform fertilizing ability. Before insemination, sperm from every male was subjected to empirical assessment under microscope at magnification of x40 using glass slide without a coverslip (Fauvel *et al.,* 2010). A total volume 1.5 ml of milt was next mixed gently and stored at 4℃ until it was used for fertilization. In each step of fertilization a batch of approximately 250 eggs from each female was gently distributed into separate small Petri dishes and then fertilized using 0.15ml of mixed milt and adding 2 ml of hatchery water continued by shaking them for 3 minutes. Later eggs were washed and 5 ml of hatchery water was poured into each Petri dish.

2.4 Incubation and examining embryo viability rates

After fertilization, the eggs were washed with hatchery water 4-5 times to remove the extra milt. To ensure eggs attachment to Petri dishes, the eggs were left for the time period of 5 minutes in those dished at room temperature. After that, plates were placed separately into small rectangular-shaped incubators (each 4.5 L capacity). Each incubator was supplied with recirculating water of temperature 20 ± 0.5 ℃ and flow rate of 1 L min^{-1}. Possibility of the occurrence of thermal shock

was reduced by adding hatchery water slowly to the Petri dishes before placing them into incubators.

The embryo survival rate was assessed to evaluate egg viability considering presence and absence of the embryo in each egg. With respect to the total number of initially fertilized eggs, after 24 hours of fertilization, embryo survival rates were calculated using the stereomicroscope (Nikon SMZ745T, Japan).

2.5 Fat extraction

The lipid fractions of the eggs were extracted with hexane-isopropanol (3:2 v/v) according to a method of Hara and Radin (1978). One gram of egg sample was homogenized with 10 ml of hexane-isopropanol mixture with an Ultra Turrax. The knife homogenization time was 30 seconds, repeated 3 times. Samples were transferred to centrifuge flasks and 6 ml of Na_2SO_4 (6.67%) was added. After shaking and releasing the pressure by opening the cap for a moment, samples were centrifuged at 5000 rpm for 5 min at 20 ºC. The upper phase of each samples was then transferred to reweighted evaporation tubes using Pasteur pipettes. This action was repeated three times, adding 1 ml of hexane before each next centrifuging. The combined upper fraction of each sample was than evaporated under nitrogen gas. The total lipid content from the eggs was then determined by weighing the tubes and calculating the proportion of lipid per g sample.

By adding 2 x 0.5 ml of hexane and vortex the sample was transferred using Pasteur pipettes to 4ml brown glass vials and stored at – 80 ºC for further use. All analyses were done in duplicates if possible.

2.6 Weight on microbalance

Before next step of the experiment all samples were weighted on microbalance in order to determine the exact concentration of the dissolved lipid. Samples were taken from the freezer and then vortexed. Approximately 1 μl of sample was weighed 3 times, using microaluminium weighing dishes and lipid concentration in the sample was calculated.

2.7 Methylation

2 mg of lipid dissolved in 500 ml hexane were used for methylation. Methylation was done with BF3 according to Appelqvist (1968). More concrete, 2 ml of dry methanol were added to the samples and heated for 10 min at 60ºC in a heating block. Thereafter samples were cooled under running water and 3 ml of BF_3 were added and mixed by vortex. Again samples were placed into the heating block for 10 min at 60 ºC. After cooling the tubes with cold water, 2 ml of NaCl 20% was added and subsequently 2 ml of hexane. All mixed by vortex and placed in the fridge for 20 minutes for separation of water and lipid phase. The upper phase was transferred by Pasteur pipettes to evaporation tubes. 1 ml of hexane was added to the samples and the action was repeated one time. The combined hexane phases were then evaporated under nitrogen gas and finally dissolved in 500 µl hexane. Samples were then transferred for storage to 2 ml brown glass vials. Methylation was checked on TLC plate. The samples after were stored at -80 ºC in the freezer until analyses.

2.8 Check on TLC

Thin layer chromatography was performed with a mobile phase containing hexane/di-etylether/acetic acid (85:15:2, v:v:v) one hour before using it. Samples after vortex were applied into the silica plate (5µl each) with a standard dot. TLC plate was placed in solvent for one hour and subsequently fatty acid methyl esters were visualized by incubation with iodine.

2.9 Analyses on GC

The fatty acid methyl esters then were analyzed with a gas chromatograph (Trace Ultra FID, Thermo Scientific) equipped with a flame ionization detector and PVT injector, using a BPX 70 column (SGE, Austin, Texas), length 50 m, id 0.22 mm, and film thickness 0.25 µm. The GC was programmed with a constant gas flow of 1.2ml/min and a temperature program which started at 70 °C for 0.5min, followed by a ramp of 30 °C/min up to 150 °C and a second ramp with a rate of 2 °C min-1 until 220 °C and a final constant time of 11 min. at 220 °C. Injector and detector

temperature were programmed at 150 °C and 250 °C respectively. The injector was programmed in split less mode, with a split less time of 0.8 min and a split flow 25 ml/min. The peaks were identified by comparing their retention times with those of the standard mixture GLC-68D (Nu-Chek Prep, Elysian, USA) and other authentic standards (Nu-Chek Prep, Elysian, USA; Larodan, Sweden).

2.10 Lipid class identification

The total lipids from eggs were analyzed with TLC to investigate major lipid class composition and phospholipid composition. The analysis was done according to Olsen & Henderson (1989) with minor changes. As a stationary phase, glass plates pre-coated with silica gel high performance TLC plates (20x10 cm; Silicagel 60; 0.20 mm layer, Merck, Darmstadt, Germany) were used. The samples were diluted to a concentration of 1µg/µl in hexane, and an amount of 5µl per sample was applied with a CAMAG TLC Sampler 4 (Camag Switzerland), 2 cm from the base edge of the TLC plates in 2 mm bands with an application speed of 250 nl/sec. Nitrogen was used as spray gas. All samples were applied in duplicate, on two plates and the distance between tracks was 10 mm.

The lipids were separated in Automated Developing Chamber (ADC 3) (Camag Switzerland) using 10 ml hexane : diethyl ether : acetic acid (85:15:2, (v:v:v) as mobile phase for major lipid classes 10 ml of a mixture of 25 ml methyl-acetate, 25 ml isopropanol, 25 ml chloroform, 10 ml methanol and 10 ml 0.25 % KCl. Plates were developed up to 8.5 cm, then automatically air dried at room temperature. Subsequently plates were sprayed manually with a solution of 3% cupric acetate in 8 % phosphoric acid and then charred for 20 min at 140 °C.

Quantitative analysis of the separated lipid classes was done by scanning the plates with a CAMAG TLC Scanner 3 (Camag, Switzerland). The scanning was performed at a speed of 20 mm/sec, and a data resolution of 100 µm/step with a slit dimension of 6.00 x 0.45 mm at a wavelength of 350 nm. Identification of the lipid classes was performed by comparison with an external standard (TLC 18-4A, Nu-Chek Prep, Elysian, USA). For data filtering, the mode Savitsky-Golay 7 and manual baseline correction were used.

2.11 Statistical analysis

Percentage of fatty acids and lipid classes from the obtained raw data as well as averages and standard deviation from all analyses were calculated in excel. Statistical evaluation was then done by using one-way ANOVA analysis and Tukey's HSD in the Statistica CZ 12 software package. The level of significance was in both cases considered at $P<0.05$ and the results are presented as mean and standard deviation.

3. Results

3.1 Embryo survival rates through ova ageing.

There was no significant difference in the embryo survival rates for eggs fertilized up to 4 hours after stripping (Fig. 3). However, the highest embryo survival percentages were obtained immediately after striping and it was 35.3 ± 11.6 % (mean ± SD). After 4 hours of in vitro storage of the eggs at 20 ºC the embryo survival rates were still 68 % of their initial rates at 0 hours post striping. Longer storage of the eggs showed significant decrease in survival rates, dropping to 5±3.7 % for the fertilization after 10 hours post striping. Important to notice is that the fish number 3 did differ significantly from the others in embryo survival rates, showing much lower results that others, close to 0 in all time intervals (Fig 4.)

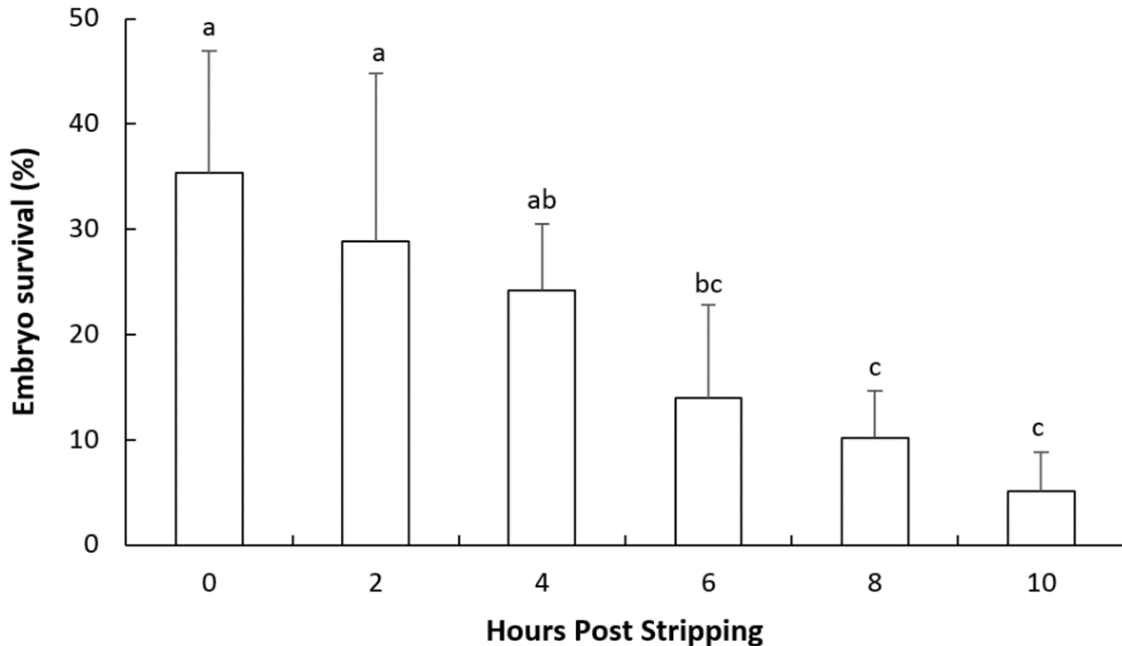

Figure 3. Effect of in vitro ova ageing on the embryo survival rates (mean ± SD). Different letters above the bars indicate significant difference between time points (P<0.05).

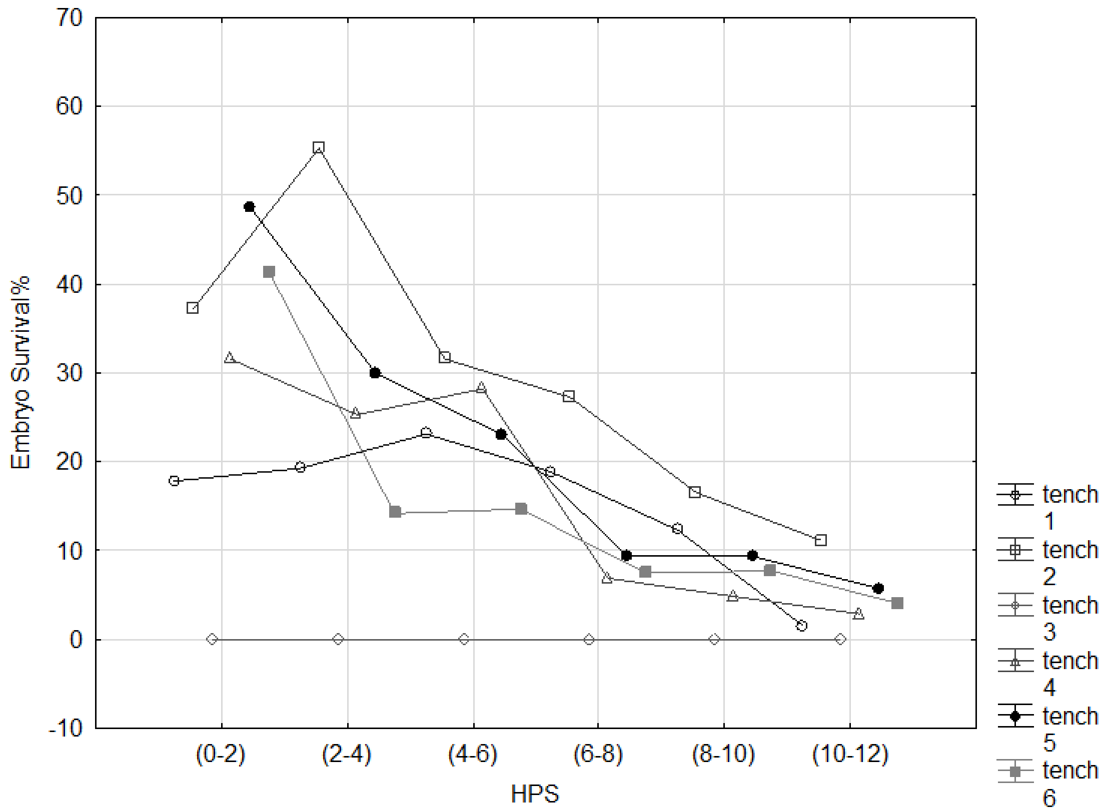

Figure 4. Fertilization rates of tench *Tinca tinca* in individual fish.

3.2 Fat content of tench eggs

- Changes over time.

The content of fat in measured samples presented in table 1 did not significantly change over time of storage. Values did not exceed more than 6.60 % in 8 hours of in vitro storage. The lowest concentration of lipid in samples was fund after 24 hours of in vitro storage and was 5.26%.

Table 1. Fat content of tench eggs during different time of in vitro storage of the eggs, values are percentages of total weight of the sample (mean ± standard deviation (SD)). The values do not show significant difference (P>0.05).

Time	Fat content %	SD
0 h	6.08	1.38
2 h	5.51	0.70
4 h	5.38	1.00
6 h	5.60	1.62
8 h	6.60	1.96
10 h	6.54	2.16
24 h	5.26	0.91

- Differences between fish

The content of the fat in tench eggs evaluated for separate individuals is presented in table 2. The smallest amount of fat was found in fish number 5 and was 4.66 % of total sampled egg mass, followed by fish number 3, with fat content being 5.15 %. The highest percentage of lipid found in tench eggs represent sample number 2, reaching 6.81 % of total sample mass.

Table 2. Fat content in tench eggs in different fishes. Values are percentage of total eggs mass (mean ± standard deviation). Note: within columns, values with different superscript letters are significantly different (P<0.05)

Fish	Fat content %	SD
tench 1	5.97^{a}	0.67
tench 2	6.81^{b}	2.82
tench 3	5.15^{c}	1.26
tench 4	6.31^{a}	1.10
tench 5	4.66^{ac}	1.06
tench 6	6.04^{ab}	0.88

3.3 Main lipid classes

- Changes in time

Investigated tench eggs during different times of in vitro storage do not show significant difference in main lipid class composition (Table 3). The highest proportions of identified lipid classes were PL, exceeding 60 % of total lipids after 2 hours of in vitro storage. Cholesterol level varied from 20.9 % in 2 hours to 22.4 % immediately after striping. The highest amount of TAG was found after 24 hours post striping and was 20.2 % of total lipids identified in the samples. Free FA were not included in evaluation as were only found in few samples.

Table 3. Lipid class composition of tench eggs during different time of in vitro storage. PhoL- phospholipids, Col- cholesterol, TAG- triacylglycerols. The values are percentage of total main lipid classes fund in eggs. Note: all values did not differ significant from each other (P>0.05).

Time/Lipid %	PhoL	SD	Col	SD	TAG	SD
0 h	59.3	2.57	22.4	1.08	18.1	1.93
2h	60.0	3.16	20.9	1.22	18.7	2.65
4 h	59.1	3.07	21.8	2.80	19.1	2.20
6 h	58.3	2.77	22.0	1.82	19.6	3.04
8 h	58.9	2.08	21.7	1.33	19.0	1.97
10h	58.7	0.55	21.5	0.57	19.5	1.03
24h	57.3	1.32	21.7	0.40	20.2	1.13

- Differences between fish

Percentage of main lipid classes was found to be similar for all fishes (Table 4). However free fatty acids (FFA) were found only in couple of samples, therefore were not included in the further evaluation. The FFA were mainly found in fish number 3 with proportion of 2.16 % of total lipid classes identified immediately after striping, 2.76 % after 2 hours, 1.51 after 6 hours, 2.20 % after 8 hours and 1.58 % after 10 hours. FFA were detected after 24 hours of in vitro egg storage in fish number 1, 3 and 6 with concentration 1.06 %, 2.76 % and 1.98 %

respectively. Cholesterol level was different in all fish eggs reached 23.12 % of total lipids identified for fish number 2 and the lowest amount was found in fish number 3 which was 19.52 %. Moreover, the content of TAG much differ between fishes and highest percentage of TAG was found in fish number 3 and was 23.43 % and the lowest 16.97 % found in fish number 5.

Table 4. Lipid class composition in tench eggs in different fishes. PhoL- phospholipids, Col- cholesterol, FFA- free fatty acids, TAG- triacylglycerols. The values are % of total main lipid classes identified in tench eggs. Note: within columns, values with different superscript letters are significantly different (P<0.05)

Fish\Lipid%	PhoL	SD	Col	SD	TAG	SD
tench 1	61.02^{a}	2.64	21.30^{a}	1.04	17.60^{a}	2.33
tench 2	55.33^{b}	2.58	23.12^{b}	1.34	20.72^{bc}	1.77
tench 3	55.66^{b}	2.82	19.52^{c}	1.28	23.43^{b}	2.24
tench 4	58.43^{ab}	1.80	22.12^{a}	1.34	19.67^{ac}	1.69
tench 5	61.22^{a}	0.66	21.71^{a}	0.85	16.97^{a}	0.28
tench 6	59.77^{a}	1.71	22.43^{ab}	0.36	17.52^{a}	1.51

3.4 Phospholipid classes composition

- Changes over time

PL composition investigated in this study for tench eggs do not show significant difference between time periods of in vitro storage (P>0.05) (Table 5). The main component of identified PL was phosphatidylcholine (PC) which reached 64.97 % after 24 hours in vitro storage and showed the smallest value of 63 % immediately after striping. Cardiolipin (CL) fractions were found in values of 16.49 % immediately after striping as the highest and 14.95 % of total identified PL in 10 hours of in vitro storage as the lowest value of CL found. Phosphatidylethanolamine (EA) did not differ much between times of in vitro storage, exceeding maximum after 8 hours post striping, which was 20.69 %.

Table 5. Phospholipid classes found in tench eggs during different time of in vitro storage. PC-phosphatidylcholine, CL- cardiolipin, EA- phosphatidylethanolamine . Note: Values are percentage of all phospholipids identified. Values within columns did not differ significantly from each other (P>0.05).

Time/Lipid	PC	SD	CL	SD	EA	SD
0 h	63.00	1.85	16.49	1.88	20.51	0.86
2 h	64.61	1.40	15.53	0.76	19.86	1.60
4 h	63.98	2.19	15.61	0.93	20.41	1.82
6h	64.35	2.87	16.14	2.21	19.51	1.39
8 h	64.69	3.32	14.95	4.32	20.69	2.73
10 h	64.69	1.86	15.28	0.37	20.02	1.68
24 h	64.97	2.42	15.55	0.96	19.49	2.06

- Differences by fish

Composition of phospholipids in eggs of tench differentiated by the individuals used in this study is presented in table 6. PC and CL concentrations do not show significant difference between studied fishes and PC reach levels of 65.64 % in fish number 3 and CL 16.93 % of total identified PL classes in fish number 4. However, EA show significant difference between fishes and the lowest level was found in fish number 3 with 18.15 % of total identified PL, exceeding highestpercentage of 21.82 % in fish number 2.

Table 6. Phospholipid composition of tench eggs in different fish. . PC-phosphatidylcholine, CL-cardiolipin, EA-phosphatidylethanolamin. Note: Values are percentage of all phospholipids identified in samples. Values with different superscript letters in a column differ significantly (P<0.05) SD- standard deviation.

Fish/Lipid	PC	SD	CL	SD	EA	SD
tench 1	63.89	1.45	15.36	0.46	20.75[a]	1.15
tench 2	62.98	2.62	15.53	3.98	21.82[a]	2.21
tench 3	65.64	2.15	16.21	1.09	18.15[b]	1.25
tench 4	63.28	2.72	16.93	2.02	19.80[ab]	1.54
tench 5	65.09	1.67	14.23	0.74	20.68[a]	1.10
tench 6	64.85	2.37	15.64	1.47	19.51[ab]	0.93

3.5 Fatty acid composition

- Changes over time

The FA composition of the oocytes and the changes over time are presented in table 8. The main fatty acids found in tench were palmitic acid (16:0), oleic acid (18:1n-9), AA (20:4n-6), EPA (20:5n-3),docosapentaenoic acid (22:5n-3) and docosahexaenoic acid (22:6n-3). The highest percentage was represented by palmitic acid, reaching 29.49 % of total FA identified in the samples in 2 to 4 hours of in vitro storage. Oleic acid was fund to be 16.98 % of total FA, 8 hours after striping. AA reached the highest level in time period 10-12 hours after striping and was 5.23 %. The percentage of EPA and docosapentaenoic acid show the highest content in time of the in vitro storage equal 10-12 hours and showing the concentration of 6.20 % and 2.33 % respectively. The DHA showed percentage of 13.70 % as the highest content at the time interval of 8 to 10 hours. Omega 3 FA found in the tench eggs reached highest percentage over time after 10 hours and was 23.96 % and omega 6 FA highest percentage was found after 4 hours reaching 10.95 %. Standard deviation is very high in some FA, especially in the sum of omega 3 FA (SD= 4.29-4.92) because fish differ much from each other in FA composition. The ratio of n-3/n-6 FA found in samples did not differ significantly over time with highest proportion of 2.32 after 10 hours of in vitro eggs storage and lowest after 8 hours – 2.06. In addition, the AA/EPA ratio did not significantly differ over time and the lowest value 0.77 was found after 2 hours of in vitro egg storage and the highest after 8 hours with value 0.98.

- Differences by fish

FA composition differed significantly between selected fish and is shown in table 9. Main FA found in eggs of different fish were: palmitic acid with highest percentage of 31.92 % in fish number 2, oleic acid (from 12.60 % in fish 3 to 19.77 % in fish 4), AA (from 4 % in fish 6 to 5.62 % in fish number 5), EPA (from 8.82 % in fish number 2 to 2.81 % in fish number 1), docosapentaenoic acid (from 3.33 % in fish number 2 to 1.14 % in fish number 1) and DHA with

highest percentage of 15.01 % in tench number 3 and lowest 12.16 % in tench number 2. Highest content of omega 3 FA was found in fish number 3 and reached 29.64 %. In comparison, fish number 1 had the lowest percentage of omega-3 FA and it was 17.13 %. Omega 6 FA had a reverse trend and showed the lowest value in fish 3 and the highest in fish 1, reaching 8.83 % and 12.52 % respectively. Therefore, it was expected and showed in results that the ratio of n-3/n-6 was 3.36 in fish number 3 representing the highest value and 1.37 in fish number 1 as the lowest value. The ratio of arachidonic acid to eicosapentaenoic acid differed greatly between fishes and was biggest in fish number 1 and reached 1.93. The lowest AA/EPA ratio was represented in samples obtained from fish number 2 and it was 0.48 (table 7).

Tab. 7. Ratios of different fatty acids in individual fish (mean). Note: AA - arachidonic acid, EPA - eicosapentaenoic acid, DHA – docosahexaenoic acid, MUFA – mono unsaturated fatty acid, LC PUFA- long chain poly unsaturated fatty acids, n-3 – omega 3 fatty acids, n-6 – omega 6 fatty acids. Note: within columns, values with different superscript letters are significantly different ($P<0.05$)

Fish/ratio	AA/EPA	AA/DHA	SFA/PUFA	MUFA/PUFA	LC PUFA n-3/LC PUFA n-6
tench 1	1.93[a]	0.44[a]	1.30[a]	1.07[a]	0.38[a]
tench 2	0.47[b]	0.34[b]	1.12[b]	0.75[b]	0.19[b]
tench 3	0.65[c]	0.34[b]	0.93[c]	0.68[c]	0.23[c]
tench 4	1.12[d]	0.34[b]	1.10[b]	0.94[d]	0.27[d]
tench 5	1.16[d]	0.38[e]	1.06[b]	0.82[e]	0.30[e]
tench 6	0.67[c]	0.31[f]	1.05[b]	0.91[d]	0.23[c]

Table 8. The fatty acid composition of tench eggs at the different times of in vitro storage. Values are percentage of total identified fatty acids (mean and standard deviations) and did not differ significantly between the different times (P>0.05). Abbreviations: SD: standard deviation, SFA: saturated fatty acids, MUFA-monounsaturated fatty acids, PUFA- polyunsaturated fatty acids, n-3 – omega 3 fatty acids, n-6 – omega 6 fatty acids.

time	time0-2	SD	time2-4	SD	time 4-6	SD	time 6-8	SD	time 8-10	SD	time 10-12	SD	time 24	SD
14:0	1.00	0.46	1.08	0.57	0.96	0.47	1.00	0.50	0.98	0.45	0.99	0.60	1.07	0.60
16:0	29.48	2.05	29.49	1.56	29.23	1.36	29.06	1.30	28.82	1.11	29.18	1.41	29.24	1.08
16:1	7.83	0.86	7.86	0.70	7.58	1.04	7.72	0.88	7.41	1.12	7.45	0.98	7.86	1.05
18:0	6.62	0.66	6.59	1.02	6.92	0.75	6.52	0.77	6.95	0.47	7.06	0.48	6.58	0.90
18:1n-9	15.80	2.53	15.74	2.62	16.49	2.88	16.23	2.68	16.98	2.54	15.56	2.80	15.59	2.54
18:1n-7	4.85	0.69	4.86	0.65	4.76	0.72	4.70	0.69	4.89	0.72	4.61	0.54	4.80	0.71
18:2n-6	5.13	1.45	5.12	1.37	5.36	1.45	5.47	1.47	5.43	1.60	4.98	1.70	5.15	1.47
18:3n-3	1.35	1.00	1.40	1.09	1.29	0.89	1.31	0.93	1.28	0.97	1.43	1.11	1.37	1.00
20:1	0.24	0.07	0.23	0.07	0.23	0.08	0.22	0.08	0.27	0.03	0.24	0.06	0.24	0.08
20:2n-6	0.76	0.18	0.76	0.21	0.77	0.14	0.76	0.13	0.82	0.15	0.73	0.16	0.76	0.16
20:4n-6	4.84	0.85	4.71	0.48	4.82	0.90	4.95	0.56	5.06	0.64	5.23	0.69	4.93	0.93
20:3n-3	0.34	0.20	0.39	0.27	0.33	0.18	0.31	0.21	0.34	0.17	0.34	0.20	0.36	0.22
20:5n-3	6.02	2.35	6.08	2.47	5.70	2.35	5.89	2.30	5.14	1.89	6.20	2.72	6.20	2.52
22:5n-3	2.29	0.82	2.25	0.86	2.13	0.84	2.19	0.90	1.94	0.73	2.33	1.02	2.29	0.85
22:6n-3	13.45	1.47	13.43	1.38	13.43	1.36	13.67	1.21	13.70	1.18	13.67	1.34	13.57	1.20
SFA	37.10	2.13	37.16	2.09	37.11	1.51	36.58	1.53	36.74	1.16	37.24	1.37	36.89	1.54
MUFA	28.72	2.55	28.70	2.57	29.06	2.87	28.87	2.56	29.55	2.34	27.86	2.68	28.48	2.91
PUFA	34.18	3.34	34.14	3.35	33.83	3.35	34.55	2.91	33.71	3.12	34.90	3.07	34.63	3.30
n-3	23.44	4.64	23.56	4.78	22.88	4.46	23.37	4.29	22.40	4.43	23.96	4.92	23.79	4.62
n-6	10.74	1.76	10.59	1.58	10.95	1.60	11.18	1.62	11.31	1.58	10.94	2.10	10.84	1.74
n-3/n-6	2.28	0.78	2.32	0.80	2.17	0.73	2.18	0.73	2.06	0.75	2.32	0.89	2.29	0.78

Table 9. Fatty acid composition of tench oocytes, showing the differences between fishes. Values are percentage of total identified fatty acids (mean and standard deviation). Values with different superscript letters within a row differ significantly - (P<0.05). Abbreviations: SD: standard deviation, SFA: saturated fatty acids, MUFA-monounsaturated fatty acids, PUFA- polyunsaturated fatty acids, n-3 – omega 3 fatty acids, n-6 – omega 6 fatty acids

Fish/Fatty acid	Tench 1	SD	Tench 2	SD	Tench 3	SD	Tench 4	SD	Tench 5	SD	Tench 6	SD
14:00	0.86[a]	0.06	0.66[b]	0.05	1.90[c]	0.14	0.61[b]	0.00	0.71[b]	0.06	1.04[d]	0.10
16:0	30.24[a]	0.33	31.09[a]	0.78	27.62[b]	0.28	28.28[b]	0.41	29.21[c]	0.67	28.46[bc]	0.61
16:1	8.18[ac]	0.31	8.03[a]	0.32	7.95[ad]	0.30	6.67[b]	0.08	6.28[b]	0.30	8.57[c]	0.40
18:0	7.46[a]	0.36	7.24[ac]	0.28	6.06[d]	0.38	7.39[e]	0.12	6.74[d]	0.45	5.87[e]	0.32
18:1n-9	18.38[a]	0.26	13.71[b]	0.09	12.60[c]	0.18	19.77[d]	0.35	17.59[e]	0.24	16.25[f]	0.14
18:1n-7	4.90[a]	0.06	4.02[b]	0.05	5.20[c]	0.07	4.12[b]	0.07	4.33[d]	0.06	5.79[e]	0.06
18:2n-6	6.34[a]	0.16	4.74[b]	0.10	2.75[c]	0.09	6.59[d]	0.09	6.09[e]	0.12	5.77[f]	0.11
18:3n-3	0.68[a]	0.02	1.13[b]	0.03	3.13[c]	0.10	0.97[d]	0.03	0.88[e]	0.03	0.98[d]	0.06
20:1	0.33[a]	0.02	0.15[b]	0.04	0.20[bc]	0.02	0.27[c]	0.03	0.25[c]	0.03	0.24[c]	0.05
20:2n-6	0.76[a]	0.02	0.50[b]	0.06	0.87[c]	0.03	0.67[d]	0.02	0.79[a]	0.02	0.95[b]	0.05
20:4n-6	5.42[ad]	0.10	4.19[b]	0.28	5.21[c]	0.18	4.80[a]	0.33	5.62[ab]	0.44	4.00[b]	0.36
20:3n-3	0.20[a]	0.04	0.25[a]	0.05	0.71[c]	0.08	0.23[a]	0.02	0.27[ab]	0.03	0.34[b]	0.03
20:5n-3	2.81[a]	0.12	8.82[b]	0.30	8.00[c]	0.11	4.27[d]	0.15	4.82[e]	0.19	6.06[f]	0.29
22:5n-3	1.14[a]	0.04	3.33[b]	0.12	2.79[c]	0.10	1.48[d]	0.06	1.73[e]	0.09	2.51[f]	0.17
22:6n-3	12.30[a]	0.25	12.16[a]	0.30	15.01[b]	0.16	13.92[c]	0.29	14.70[b]	0.22	13.20[d]	0.38
SFA	38.57[a]	0.47	38.99[a]	0.60	35.59[b]	0.33	36.27[b]	0.53	36.66[c]	0.45	35.36[b]	0.69
MUFA	31.78[a]	0.18	25.91[b]	0.40	25.94[b]	0.11	30.82[c]	0.53	28.45[d]	0.35	30.84[c]	0.39
PUFA	29.65[a]	0.59	35.10[b]	0.98	38.47[c]	0.34	32.91[d]	0.83	34.89[bd]	0.78	33.80[bd]	1.03
n-3	17.13[a]	0.42	25.68[b]	0.68	29.64[c]	0.30	20.86[d]	0.48	22.39[e]	0.44	23.09[e]	0.79
n-6	12.52[a]	0.18	9.42[b]	0.31	8.83[c]	0.10	12.05[a]	0.35	12.50[a]	0.35	10.71[d]	0.27
n-3/n-6	1.37[a]	0.02	2.73[b]	0.03	3.36[c]	0.04	1.73[d]	0.01	1.79[e]	0.02	2.16[f]	0.04

4. Discussion

With extended periods of time after ovulation, oocyte in II stage metaphase experiences deterioration in quality referred to as post-ovulatory oocyte ageing. Oocyte ageing and over-ripening in fish is an important issue both in nature and under farming conditions. Unfortunately available information about oocyte ageing in nature and its effects on fish reproductive behaviour is limited. Although the physiological changes of post-ovulatory oocyte ageing have been studied in broad scale, the molecular mechanisms controlling this process are still not well defined (Lord *et al.*, 2013).

Also changes of lipid content and composition during oocyte over-ripening have been poorly studied and the main information about influence of lipid content and composition is focused on oocyte quality aspects. It has been shown that oxidative stress is one factor during oocyte over ripening (Lord *et al.,* 2013; Samarin 2015) and this could then subsequently lead to lipid oxidation and a changed content/composition of lipids during over-ripening. Therefore the aim of the present work was to evaluate lipid composition in oocytes of tench during over-ripening and correlate it to fertilization ability.

Hence the first goal off this study was to observe the time of over-ripening in tench. To do so, it is important to correlate all changes that occurred inside oocyte and notice the time point of tench eggs when the fertilization ability was significantly decreasing, which in this case was gradually decreasing with time points of observation.

The optimal time period for egg collection has been studied and determined for most popular farmed fish from *Salmonidea* and *Cyprinidae* family. The maximum time period between ovulation and deterioration of the egg quality for some species are for example: for Common carp *(Cyprinu scarpio)*- 50-80 minutes, Grass carp *(Ctenopharyngodon idella)* 35-45 minutes, Rainbow trout up to 7 days and for sturgeons *(Acipenser sp.)* about 2 hours (Rottman, 1991). In many other species it has to be yet investigated. It has to be taken into account that the time period between ovulation and over-ripening of the eggs is in great manner affected by temperature. Therefore it is proposed to use terms of degree-hours or degree-days (depending on

the fish species) because in this way is more practical and comparable for mass production in hatcheries.

The optimal time periods for actions mentioned above in tench are still under investigation. Flaishans *et al.* (2007) showed that *Tinca tinca* should show 30% of fertilization rate after 5 hours in vitro egg storage, which in case of this fish is consider normal fertilization rate in captivity. In the present study fish fertilization rates were obtained on level 35 % immediately after stripping, gradually decreasing in time and reaching 10 % after 8 HPS. After 24 hours the rates were close or equal to zero for all fishes. Fish number 3 however, showed 0 % of fertilization rates during all time periods of in vitro storage. In general, in this study all 6 fishes differed in fertilization rates (Fig. 4).

Evaluated lipid content of the tench eggs did not differ significantly during prolonged in vitro storage of the eggs, indicating that a changed fat content did not play a role in oocyte over-ripening. However fat content was significantly different for individuals chosen for the experiment, indicating some differences in individual metabolism and incorporation of the lipids to ovary and eggs of this fishes. Tench number 3 which had the lowest fertilization capacity had an intermediate fat content, hence it seems fat content was not a factor for the decreased fertilization capacity in this case either. In comparison to our work, studies performed on perch *Perca fluviatilis,* showed much lower fat contend of the eggs (approximately 2 %) that did as well not correlate with fertilization rates (Żarski *et al*., 2017).

There are not many studies performed on the fish regarding fat content and its correlation to fertilizing ability. However Wu *et al*. (2010) showed that higher lipid contend of rat oocytes did negatively affect fertilization rates. It is also known, that lipid content in fish is species specific and fish with longer time between spawning and hatching of the eggs will have higher reserves of lipids inside the egg (Kaitarana and Ackman, 1994). For Japanese sardine *Sardinops sagax*, a positive correlation between the lipid content of female muscle and that found in the ovary was reported by Morimoto (1991), who later showed that female sardine with a higher muscle lipid content had larger ovaries and produced more eggs that had a higher yolk content than did females with a lower muscle lipid content (Morimoto, 1996). In conclusion, we can propose for future studies to investigate the influence of temperature and fat incorporation to tench oocytes as

well as optimum level for tench in case of fat content of eggs that not have detrimental effect on fertility abilities.

Also studies performed on human showed significant correlation with total amount of fat percentage of body mass of women and decrease in fertility abilities of their oocytes (Mulders *et al*., 2003). The accumulation of intracellular lipid leads to high levels of FFA concentration. FFA are especially sensitive to oxidation and the damage that its causing with increasing the levels of cytotoxins and highly reactive lipid peroxides which ultimately are detrimental to intracellular organelles, particularly the endoplasmic reticulum and mitochondria (Malhi H *et al*., 2008). However we must consider that fish oocytes and its maturation differ greatly with other species of animals and oocytes differ among the species of the fish as well.

Investigated main lipid classes in this study did not show significant difference over time intervals (Tab. 3). PL were found in the highest proportion of main lipid classes reaching the value of 60 % of total identified main lipid classes in tench eggs. PL, especially PC are found to be main component of the eggs of cod and many other marine species (Pickova *et al.,* 1997) which was similar in tench egg composition in the present study. In species with lower content of TAG in eggs, PL play an important role not only as a structural component of membranes but as well as important source of energy during embryonic development. In this study levels of PL in fish eggs did not differ with different fertilization rates, as they were similar for all fishes where fertilization rates show much bigger range.

Cholesterol level investigated in this study differed much among individuals but did not change over time (Tab 4). The same tendency was represented by TAG, although there is no significant but a slight increase of TAG over time. TAG and FA are present in the follicular fluid of numerous animal species and there is growing interest reaching for understanding how these substrates are ultimately utilised by ovarian somatic cells as well oocytes for energy production (Dunning, 2014).

Using an array of techniques, many studies have quantified lipids in oocytes and showed that TAG is the major component for most of the species (Sturmey *et al*., 2009). In opposite to those findings, we found PL to be the main constituent of the lipid fraction in tench oocytes.

In this study cholesterol levels were lowest in fish number 3 which also had lowest fertilization rate as well highest level of TAG. Fish number 5 with the highest fertilization rate at 0-2 HPS on the other hand had the lowest percentage of TAG and quite high levels of cholesterol (21.71 % of total identified main lipid classes). This could indicate some connection of fertilization rate and the levels or ratio of TAG and cholesterol. However we have to keep in mind that these are just single fishes and further studies with more fish should be conducted to evaluate this hypothesis. However, fish number 3 as well as showed as the only fish FFA during most time periods, indicating some early enzymatic breakdown of lipids within the oocytes. The reason for this remained unclear.

FFA were only detected in a few samples and mainly the later stages of storage (24 HPS) indicating that they were most probably a product of enzymatic breakdown during prolonged storage and therefore a sign of deterioration.

Most authors have suggested that oxidative stress and increased level of reactive oxygen species play important role in oocyte ageing. (Samarin *et al,* 2015). During oxidation, membrane lipids of mitochondria are especially sensitive (sphingomyelin and CL) and any disruption of mitochondrial functionality will be shown as decrease in detectable CL (Paradies*et al*., 2009). That is due to their high content of PUFA. Disruption of mitochondrial functionality may lead to apoptosis of the cell. In this study however we did not noticed any significant decrease of CL or other PL classes over time or between individuals. That indicates in this case the lipid oxidation was not the factor strongly contributing to oocyte ageing.

Still very little is known about effects of poor feeding regimes or low level of female fat reserves influencing the fish in the wild, although many studies were performed on farm fish in turns of broodstock diet and effects on egg FA composition impacting the egg quality and reproductive success (e.g. Bell *et al*., 1997; Almansa *et al*., 1999; Bruce *et al*., 1999; Izquierdo *et al*., 2001; Mazorra *et al*., 2003; Tveiten *et al*., 2004 ; Salze *et al*., 2005). Alteration of the diet can affect yolk lipid composition, which was already proven for salmon (Wiegand, 1996) and therefore contribute to change in fertilization rates.

It has to be considered that marine fishes studied to date have considerably low or no ability to convert C_{18} precursors to 20:5n-3 and further to 22:6n-3 due to lack or low content of Δ5-desaturase (Bell 1996, Tocher *et al*., 1992), therefore it is necessary to distinguish these differences in the lipid metabolism of fishes. Tench as a fresh and brackish water fish should therefore be compared to fresh water fishes, especially form *Cyprinidae* family like zebrafish *Danio rerio.* For example Jaya-Ram (2008) showed FA profiles of the eggs were corresponding to dietary treatment where increased linseed oil lowered the deposition of DHA, EPA and AA in fish tissues and DHA in fish eggs. This also influenced positively egg production and hatching rates in zebrafish were obtained ratio of n-3/n-6 was balance and equal 8.28±0.42.

In case of tench ratio n-3/n-6 were 3.36 for the fish number 3 with the lowest fertilization rate and the lowest ratio 1.37 for the fish number 1 that showed intermediate fertilization rate (Fig. 4, Tab. 9). Although in this study ratio n-3/n-6 was not directly connected to fertilization rates we can notice some indications of its importance for obtaining the best fertilization rates. It is essential for further studies to investigate and optimize proper ratio of n-3/n-6 in tench that is corresponding with best fertilization and hatching rates of this fish. It has to be taken into account that the n-3/n-6 ratio in the natural diet of all organisms is different in fresh and marine environment, being much higher in marine compared to freshwater (Arts *et al.,* 2001; Henderson and Tocher 1987; Kaitaranta and Linko 1984; Pickova, 1997), which will subsequently influence the deposition of FA into eggs.

Eggs of Atlantic salmon *(Salmo salar)* investigated by Pickova *et al.* (1999) with regard to lipid composition of farmed and wild females show a significant difference between FA composition and hatching rate between the eggs of fish of different origin. Achieved hatching success of wild stock was >95% while cultured fish showed 40-75 % of successful hatching. The egg lipids from farmed female salmon were richer in omega 3 FA, compared to lipid composition of free living females. Survival was significantly affected by imbalance of n-3 and n-6 FA. In PL fraction EPA was two times higher in eggs of the wild population. In the present study in tench, percentage of EPA differed much among the individuals, reaching 8 % in fish number 3 and in fish number 1 were only 2.81% of EPA was found. However EPA could not be connected to fertilization rates nor do change significantly over time. This conclusion was found for all FA investigated in this

study and to compare ratios and their effect on oocyte quality had to be done using studies performed on different fishes.

Similar studies performed on fish with more pronounced freshwater history were done by the same author. Pickova *et al.,* (2005) investigated composition of Arctic char *(Salvelinus alpinus)* eggs with addressing the importance of eiocosapentaenoic acid, DHA and AA as well as their ratios in fish reproduction. The author agreed that the ratio between AA and EPA will be crucial for many physiological functions, depending on the species and its biology as well evolution of the species. AA/EPA ratio calculated for wild stock was 0.83 in PL fraction of the analysed eggs and 0.18 and 0.21 in farmed fish. The broodstock of Arctic char in the study show 60-80 % of fertilization success in wild population and 30-40 % in farmed fish. In present study tench show similar value of the ratio reaching 1.93 for fish number 1 and 0.47 as the lowest for fish number 2 (table 7). In addition, AA/DHA ratio in tench did not show great variance and the values were 0.35±0.09. AA/EPA and AA/DHA ratios in case of tench did not showed connection to survival rates as well did not change greatly over time.

Moreover the study performed on Arctic char indicated that this fish has very limited elongation and desaturation ability because the ratio between long-chain PUFA (LCPUFA) (with more than 20 carbons) of n-3 and n-6 was significantly more altered then n-3 and n-6 PUFA (Pickova *et al.,* 2005). In Arctic char the ratio of LCPUFA n-3/LCPUFA n-6 were 4.0 for wild and 31.3 and 26.5 in cultured fish. In present study tench showed much lower variation between individuals, from 0.19 in fish number 2 to 0.38 in fish number 1 (table 7). The ratio of LCPUFA n-3/LCPUFA n-6 did not change over time as well.

5. Conclusion

Until now, reports have indicated that oxidative damage does contribute to ageing of the oocyte (Samarin *et al.,* 2015). Most authors have suggested that oxidative stress and increased level of reactive oxygen species are somehow involved in oocyte ageing. However in this study lipid content and composition did not change significantly over time indicating another reason of deterioration of oocyte quality than lipid oxidation. As well, main and PL classes did not show any significant changes over time that could lead us to new assumption of reason about decrease of oocyte. FA composition of tench eggs as well could not be connected to prolonged in vitro storage of tench eggs. However individual fishes in this study showed much different fertilization rates that could be linked with their levels of cholesterol and TAG in a minor degree. This topic still remains not well understood. It is therefore necessary for further studies to include more individuals in investigations. As well as it is of great importance taking into account environmental factors like temperature of egg storage or photoperiod and genetic structure of the population as they contribute to specific incorporation of lipids and FA into the ovary and eventually oocytes. It would be beneficial for further studies to investigate this topic more broadly.

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7. List of abbreviations

FA- fatty acid

ROS - reactive oxygen species

PUFA - poly unsaturated fatty acids

HUFA - highly unsaturated fatty acids

MUFA – mono unsaturated fatty acids

LC PUFA- long chain poly unsaturated fatty acids

DHA – docosahexaenoic acid

EPA - eicosapentaenoic acid

LA - alfa linoleic acid

AA - arachidonic acid

SFA - saturated fatty acids

TAG – triacylglycerol

PL - phospholipid

PC - phosphatidylcholine

PE – phosphatidylethanolamine

CL - cardiolipin

HPS - hours post stripping

CPH - carp pituitary hormone

TLC - thin layer chromatography

GC - gas chromatography

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9. Abstract

Thesis title: Variability of lipid classes and fatty acid composition during over ripening of oocytes from tench (*Tinca tinca*)

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Study program: Master study program

Field of study: Agricultural specialization/Fisheries and Protection of Waters

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In this project, the eggs of tench *(Tinca tinca)* were used for the evaluation of changes in fatty acid (FA) composition and lipid classes during *in vitro* ova ageing until the occurrence of over-ripening. Stripped ova of 6 females were collected separately and stored in sterile cell culture plates in an incubator at 20 °C for up to 24 hours post stripping (HPS). Stored ova were fertilized at 0, 2, 4, 6, 8, 10 and 24 HPS. Egg eyeing and hatching rates were assessed as indices of egg quality. Upon fertilization at each HPS, samples were taken separately frozen in liquid nitrogen and stored at -80°C till further analyses. Differences in the FA and lipid composition and embryo survival rates at the different fertilization times post-stripping were evaluated.

The lipid fraction was extracted from the oocytes according to Hara and Radin (1978). The lipid content of the samples was determined gravimetrically from total extracted lipid. The FA were then analysed with a gas chromatograph equipped with a flame ionisation detector and PVT injector. The peaks were identified by comparing their retention times with those of authentic standards. Major lipid classes and the phospholipid classes were separated according to (Olsen and Henderson, 1989). Quantitative analysis of the separated lipid- and phospholipid classes was done by scanning the plates after derivatisation with a CAMAG TLC Scanner 3 (Camag, Switzerland). Identification of the lipid classes was performed by comparison with authentic standards applied on the same plate.

In present study no significant changes were observed in lipid content and composition during storage of eggs until over-ripening. As well FA composition did not change over time indicating another reason of deterioration of oocyte quality than the proposed lipid oxidation. However individual fishes in this study showed much different fertilization rates that corresponded with their levels of cholesterol and triacylglycerols in minor degree.

Key words: Oocyte ageing, Over-ripening, Tench, *Tinca tinca*, Egg quality, Lipid composition, Fatty acid composition, Reproduction

10. Abstract in Czech

Název diplomové práce: Variabilita tříd lipidů a složení mastných kyselin během přezrání oocytů z řas (Tinca tinca)

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V rámci této práce byly sledovány změny v zastoupení mastných kyselin (MK) a hlavních tříd lipidů během procesu zrání jiker u lína obecného (*Tinca tinca*). Experiment byl prováděn in vitro a to až do výskytu známek přezrávání jiker. Vytřené jikry od šesti jikernaček byly umístěny v sterilních kultivačních miskách do inkubátoru při teplotě 20°C. Jikry umístěné v inkubátoru byly postupně oplodňovány a to 0, 2, 4, 6, 8, 10 a 24 hodin po výtěru. Jako ukazatel kvality použitých jiker byla u jednotlivých skupin sledována míra dosažení očních bodů a líhnutí plůdku. Vzorky byly odebírány po oplodnění jiker v jednotlivých časech, zmraženy tekutým dusíkem a udržovány při teplotě -80 °C do provedení analýzy. Rozdíly v zastoupení mastných kyselin a hlavních tříd lipidů spolu s mírou přežití embryí vyvíjejících se v jikrách oplodněných v různém časovém odstupu od výtěru byly vyhodnoceny a jsou prezentovány v rámci této práce.

Extrakce lipidové frakce z jiker byla provedena dle Hara a Radin (1978). Podíl lipidů ve vzorcích byl stanoven gravimetricky z celkových extrahovaných lipidů. Mastné kyseliny byly posléze analyzovány metodou plynové chromatografie spojené s plamenovým ionizačním detektorem. Píky cílových látek byly identifikovány na základě porovnání jejich retenčních časů s retenčními časy použitých analytických standardů. K oddělení fosfolipidů a jednoduchých lipidů byla použita metoda dle OlsenaHenderson(1989). Zastoupení jednotlivých skupin lipidů bylo poté kvantifikováno skenováním inkubačních misek pomocí CAMAG TLC Scanner 3 (Camag, Švýcarsko). Identifikace jednotlivých skupin lipidů byla opět realizována na základě porovnání s autentickými standardy nanesenými na stejnou inkubační misku.

V rámci studie nebyly pozorovány žádné statisticky významné změny v obsahu a složení lipidů u jiker inkubovaných po výtěru až do známek přezrání. Výsledky tohoto experimentu indikují, že zhoršování kvality jiker po výtěru má jiný důvod, než předpokládaná oxidace buněčných lipidů, protože jejich obsah a zastoupení jednotlivých skupin se s časem zrání jiker neměnil. Přestože jednotlivé ryby v rámci této studie vykazovaly velmi odlišné míry oplodnění, tyto korelovaly s obsahem cholesterolu a částečně i triacylglycerolů těle těchto jednotlivých ryb.

Klíčová slova: Zrání jiker, Přezrání, Lín, *Tinca tinca*, kvalita jiker, Zastoupení lipidů, Zastoupení mastných kyselin, Rozmnožování