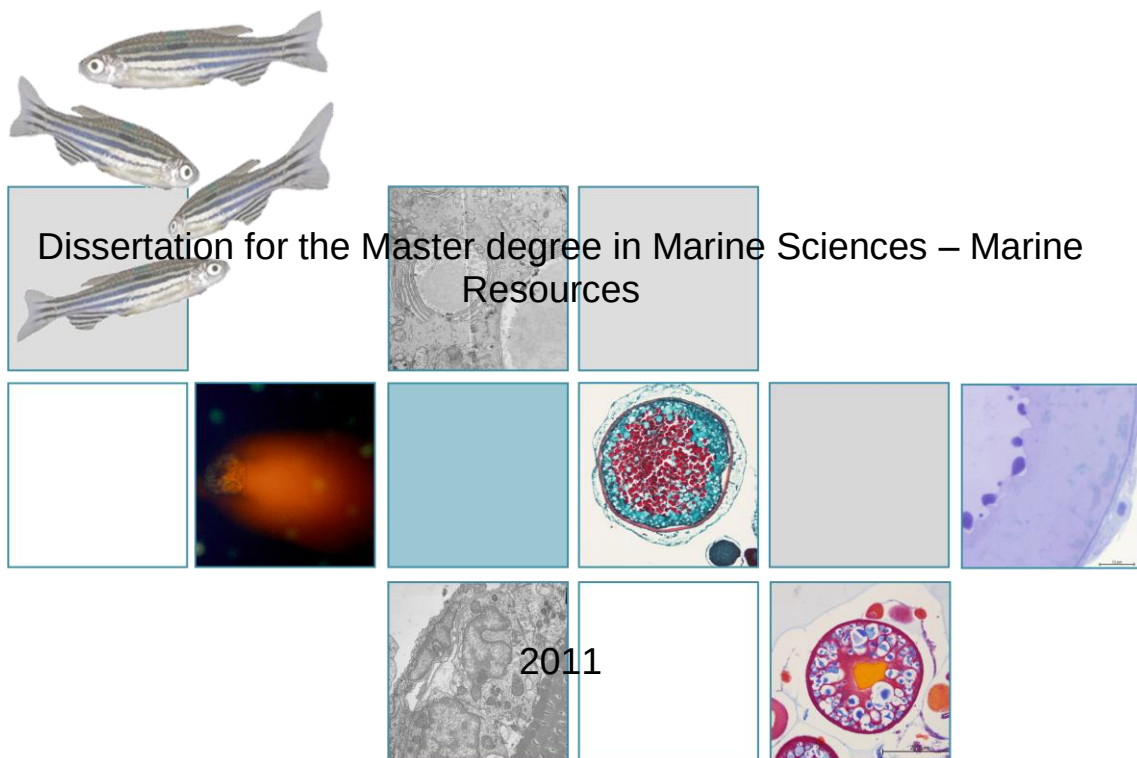


***In vitro* modeling of the oocyte development in zebrafish (*Danio rerio*): the role of hormones in maturation, apoptosis and sex-reversal of oocytes at different development stages**

Maria Lígia da Silva Sousa



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Dissertation application to the master degree in Marine Sciences - Marine Resources submitted to the Institute of Biomedical Sciences Abel Salazar, University of Porto

Supervisor: Doctor Ralph Urbatzka
Auxiliary Researcher
Centre for Marine and Environmental Research (CIIMAR)

Co- Supervisor: Professor Maria João Rocha
Associate Professor
Institute of Biomedical Sciences Abel Salazar, University of Porto and
High Institute of Health Sciences – North (ISCS-N)

U. PORTO



INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO



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In vitro modeling of the oocyte development in zebrafish (*Danio rerio*): the role of hormones in maturation, apoptosis and sex-reversal of oocytes at different development stages

“Vai ao encontro da sabedoria

Como quem lavra e semeia”

Livro de Ben-Sira, 6:19

In vitro modeling of the oocyte development in zebrafish (*Danio rerio*): the role of hormones in maturation, apoptosis and sex-reversal of oocytes at different development stages

Contents

Acknowledgements.....	ix
Abbreviations.....	xi
List of Tables.....	xiii
List of Figures.....	xiii
Resumo.....	xv
Summary.....	xvii
Introduction.....	1
Oogenesis as a conserved process in the evolution of vertebrates.....	1
Morphological changes during oocyte development.....	2
Endocrine regulation of oocyte development.....	4
Importance of the early stages.....	6
State of knowledge of endocrine regulation of early stages.....	7
Zebrafish as model.....	9
Aims of the study.....	10
Methods.....	13
Animal maintenance.....	13
Isolation of ovarian follicles and <i>in vitro</i> Culture.....	13
Viability test.....	13
Light microscopy.....	14
Contamination control.....	14
Paraffin sections.....	14
Goldner Trichrome Staining (Goldner, 1938).....	15
Azocarmine & Aniline staining – AZAN (Romeis, 1948).....	15
Immunohistochemistry.....	16
Electron microscopy.....	16
Alkaline Comet Assay.....	17
<i>In vitro</i> Exposure Experiment.....	18
Stereology.....	18
Statistical analysis.....	19
Results.....	21
General characterization of Oocytes stages.....	21
Imunohistochemistry assays.....	26
Cell culture conditions.....	27
Viability and Morphological alteration during Oocyte culture.....	29

Exposure to Reproductive and Stress Hormones	38
Stereology	46
DNA damage: Comet assay	48
Discussion	51
Viability and Morphological Characteristics During The <i>In vitro</i> Culture	51
Immunohistochemistry optimization.....	53
Ultrastructural characterization of oocytes exposed to the different hormones	54
Stereology	58
Comet assay	60
Conclusions	63
References	67

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ABBREVIATIONS

“n” – Nuage(s)

11KT - 11- Ketotestosterone

ACTH - Adrenocorticotrophic hormone

AD – Adheren junction(s)

Apes - 3-Aminopropyltriethoxysilane

AZAN – Azocarmnie & aniline staining

bl – Basal lamina

CA - Cortical alveoli

cAMP – Cyclic adenosine
monophosphate

Cort – Hydrocortisone

ctrl - Control

DHP - 17, 20 β -dihydroxy-4-pregnen-3-one

DMSO – Dimethylsulfoxide

DNA - Desoxiribonucleic acid

E₂ - 17 β - Estradiol

EDTA - Ethylenediaminetetraacetic
Acid

EGF – Epidermal growth factor

ER – Endoplasmic reticulum

Fig – Figure

FSH or **GTH-I** - Follicle Stimulating
hormone

FSH β – Subunit β of FSH

G – Granulosa cell/layer

G0 – Quiescent phase of cell cycle

G1 – Gap 1 interphase of cell cycle

G2 – Gap 2 of interphase of cell cycle

Ga- Golgi apparatus

GJ – Gap junction(s)

GnRH – Gonadotropin-Releasing
Hormone

GTH - Gonadotropin hormone(s)

GV – Germinal vesicle

GVBD – Germinal vesicle break down

H&E - Hematoxilyn & Eosin staining

H₂O₂ – Hydrogen peroxyde

hCG – Human chorionic gonadotropin

HCl – Hydrogen chloride

HPG –Hypophalamus – pituitary –
gonad

IGF- I – Insulin growth factor I

IGF-II – Insulin growth factor II

IHC - Immunohistochemistry

L-15 – Leibovitz L-15 medium

LH or **GTH-II** - Luteinizing Hormone

LH β – Subunit β of LH

LM – Light microscopy

LMA – Low melting point agarose

MC2R – Melanocortin 2 receptor

MIS or **MIH** - Maturation inducing steroid

mit – Mitochondria

MPF –Maturation promoting factor

mPr - Membrane Progesterone receptor

mRNA – Messenger RNA

MVB –Multivesicular bodies

NMA – Normal melting point agarose

nPr - Nuclear Progesterone receptor

OMC – Oocyte maturation competence

PBS - Phosphate buffer solution

PGC – Primordial Germ Cells

pn – Perinucleus

PSI – Point-sample intercept

rER – Rough endoplasmic reticulum

RNA - Ribonucleic acid

rpm – Rotations per minute

S phase – Synthesis phase of interphase of cell cycle

sER – Smooth endoplasmic reticulum

Star – Steroidogenic acute regulatory protein

Sv – surface density

T – Thecal cell/layer

TEM – Transmission Electron Microscopy

TGF β – Transforming growth factor β

TJ –Tight junction(s)

Tris- Tris (hydroxymethyl) aminomethane

ve - Vitelline envelope

Vtg – Vitellogenin

v_v – Volume weighted mean value

y – Yolk

zp – Zona pellucida

LIST OF TABLES

Table 1 Contingency analysis of morphological alterations observed under TEM in oocytes of stage I.....	40
Table 2 Contingency analysis of morphological alterations observed under TEM in oocytes of stage II.....	41

LIST OF FIGURES

Fig. 1 Oogonia	21
Fig. 2 Stage I	22
Fig. 3 Stage II	23
Fig. 4 Stage III	24
Fig. 5 Stage IV.....	24
Fig. 6 Different histological staining.....	25
Fig. 7 Controls of IHC	26
Fig. 8 IHC of oocytes after 2 days of culture.	27
Fig. 9 Histology images (H&E) of the separation of the oocytes.....	27
Fig. 10 Vitellogenic oocytes cultured at different medium concentrations.....	28
Fig. 11 Diameter of vitellogenic oocytes after culturing in different concentrations of Leibovitz L-15 medium.....	28
Fig. 12 Contamination detection.	29
Fig. 13 Graphic representing the viability observed in the time course of isolated oocytes by paraffin histology and trypan blue staining test.....	29
Fig. 14 Trypan blue viability test.....	30
Fig. 15 Histological evaluation of oocyte viability (H&E).	30
Fig. 16 Histology results of the time course with isolated oocytes at stage I, II and III/IV (H&E).....	31

Fig. 17 LM of an isolated vitellogenic oocyte with overnight of post fixation.	32
Fig. 18 LM of isolated oocytes of zebrafish cultured for 4 days	32
Fig. 19 LM of the fragments of zebrafish ovaries cultured for 4 days.....	33
Fig. 20 Presence of lipid droplets in follicular cells.	34
Fig. 21 TEM of oocytes at stage I of the time course.....	35
Fig. 22 TEM of stage II oocytes of the time course	36
Fig. 23 TEM of vitellogenic stages along 4 days of culture	37
Fig. 24 Goldner staining of oocytes.....	38
Fig. 25 Periphery of oocytes at stage I exposed for 2 days to the set of the chosen hormones.....	42
Fig. 26 Inside of oocytes at stage I exposed for 2 days to the set of the chosen hormones	43
Fig. 27 Periphery of oocytes at stage II exposed for 2 days to the chosen hormones	44
Fig. 28 Inside of oocytes at stage II exposed for 2 days to the chosen hormones.....	45
Fig. 29 Results of the nuclear weighted mean value of granulosa cells exposed to the different hormones.....	46
Fig. 30 Differences in the nuclear Vv of granulosa cells in control oocytes.	47
Fig. 31 Surface density of oocytes of stage I and II exposed to the different hormones. ..	47
Fig. 32 Examples of comets of the validation procedure	48
Fig. 33 Validation of Comet assay results	49
Fig. 34 Example of a comet of a stage II oocyte measured by CometScore™.....	49
Fig. 35 Comet assay for oocytes exposed to different hormones	50

RESUMO

A regulação endócrina do desenvolvimento dos ovócitos é um processo fascinante onde muito ainda há por desvendar. As células germinativas primordiais diferenciam-se segundo a ativação em cascata de determinados genes e uma sinalização apropriada de esteroides. A fase de crescimento ocorre sob controlo da hormona foliculoestimulina (FSH) que estimula a produção de 17β -estradiol (E_2) que por sua vez promove a vitelogénese. Após um crescimento acentuado quer do tamanho dos ovócitos quer do seu conteúdo dá-se a maturação final que é iniciada pela hormona luteinizante (LH) seguida da ação da hormona indutora da maturação (MIH). Todos estes sinais hormonais operam por vias endócrinas/parácrinas que envolvem também outros fatores e péptidos locais.

Muitos estudos têm-se focado na vitelogénese e na regulação da maturação final e pouco se sabe sobre os estágios primordiais dos ovócitos. Por isso, o trabalho que se segue analisa os efeitos de algumas hormonas e da adenosina monofosfato dibutil cíclica (cAMP) no desenvolvimento dos ovócitos de peixe zebra (*Danio rerio*) e respetivas células foliculares em diferentes estágios de desenvolvimento. Os fenómenos de apoptose também foram objeto de estudo.

Iniciou-se o trabalho com o estabelecimento de uma cultura de folículos *in vitro* sem adição de soro sendo a sua viabilidade verificada utilizando vários métodos, - teste do azul tripano, microscopia de luz (LM) e microscopia eletrónica de transmissão (TEM). Nestas condições, os ovócitos apresentaram atividade intracelular normal após dois dias de cultura e os primeiros sinais de degradação foram observados ao dia 4 usando TEM, sendo os ovócitos vitelogénicos os mais afetados.

De seguida foram escolhidas seis hormonas para uma exposição *in vitro* curta e aguda. Foram testadas três hormonas sexuais, E_2 , 11-ketotostosterona (11KT), $17, 20 \beta$ -dihidroxi-4-pregnen-3-one (DHP) e da cAMP que mimetiza a ação das gonadotropinas. Para além destas, foram também testadas duas hormonas envolvidas nos processos de stress como a hidrocortisona (Cort) e a hormona adrenocorticotrófica (ACTH).

Foram analisadas e registadas as alterações morfológicas dos ovócitos e células foliculares nos diferentes estágios de desenvolvimento, com LM, TEM e análise estereológica. Cort induziu a proliferação do retículo endoplasmático (ER) em ovócitos no estágio I enquanto que E_2 , cAMP e Cort tiveram esta ação no estágio II -ER em torno dos

alvéolos corticais (CA), retículo endoplasmático liso (sER) e retículo endoplasmático rugoso (rER), respetivamente. Interessante foi o efeito da ACTH que resultou numa degradação generalizada dos ovócitos. 11KT, DHP e Cort também parecem afetar o desenvolvimento das células foliculares, no entanto são necessários mais estudos que nos permitam confirmar estes resultados.

Paralelamente, desenvolveu-se um protocolo de ensaio cometa para ovócitos de peixe zebra de modo a avaliar o efeito das hormonas e cAMP sobre o ADN dos mesmos. Os resultados obtidos revelaram uma elevada degradação do ADN nas exposições a ACTH e Cort.

Pela primeira vez, demonstrou-se uma ação direta da ACTH nos ovócitos, verificando-se a degradação e apoptose da célula e por isso uma evidência da função do recentemente descoberto recetor da ACTH nas gónadas de peixe-zebra.

A combinação de todos os resultados obtidos revela novas perspetivas dos efeitos das hormonas nos estágios iniciais de desenvolvimento dos ovócitos e na apoptose. Estes resultados podem ser úteis para melhor compreender a ovogénese.

SUMMARY

The endocrine regulation of oocyte development is a fascinating process, where many aspects are still unknown. The primordial germ cells differentiate under a cascade of early genes activation and appropriate steroid signalling into oogonia. The growth phase of the oocytes is under the control of follicle stimulating hormone (FSH) that stimulates the production of 17 β -estradiol (E₂), which in turn induces vitellogenesis. After a strong increase in size and oocyte contents, the maturation is initiated by luteinising hormone (LH) and the subsequent action of the maturing inducing hormone (MIH). All those hormonal signals operate in an endocrine/ paracrine manner that involves various local peptide factors.

Many studies focused so far on the vitellogenesis and the regulation of the final maturation, whereas little is known about early oocyte stages. Therefore, the following work analyzed the effects of several hormones and of the dibutyryl cyclic adenosine monophosphate (cAMP) on the development of the early stages of zebrafish (*Danio rerio*) oocytes and respective follicular cells. Apoptosis phenomenons were also studied.

At first a serum-free *in vitro* culture of oocyte-follicle complexes was established and the viability assessed by various methodologies - trypan blue test, light microscopy (LM) and transmission electron microscopy (TEM). Under the established conditions, the oocytes showed normal intracellular activity after 2 days and the first signs of degeneration were observed after 4 days of culture by TEM, especially in vitellogenic oocytes.

Following this, six hormones were chosen for a short acute *in vitro* exposure. Three sexual steroids included E₂, 11-ketotestosterone (11KT), 17, 20 β -dihydroxy-4-pregnen-3-one (DHP), and (cAMP) as a mimic of gonadotropin action. Furthermore, two hormones involved in stress processes were also used, the hydrocortisone (Cort) and the adrenocorticotrophic hormone (ACTH).

The oocytes and follicle cells of different developmental stages were analysed for morphological alterations with LM, TEM and stereology, and several effects were observed. Cort induced the proliferation of the endoplasmic reticulum (ER) in stage I oocytes, while E₂, cAMP and Cort had this effect on stage II oocytes (ER around cortical alveoli (CA), smooth endoplasmic reticulum (sER) and rough endoplasmic reticulum (rER), respectively). Surprisingly, ACTH exposure resulted in widespread oocyte degradation. 11KT, DHP and Cort also seem to affect the development of follicular cells,

however further investigations are needed to complement these results. Furthermore, a protocol for a comet assay with fish oocytes was developed. An exposure of oocytes at stage I and II to the same hormones, revealed a high degree of DNA degradation in the ACTH and Cort groups.

For the first time, a direct action of ACTH was demonstrated on oocytes by inducing cell degradation and apoptosis, and therewith evidence for the function of the recently discovered receptor for ACTH in zebrafish ovaries.

The combination of all the results of the present work showed new insights about the effects of hormones on the early stages of oocyte development and on oocyte apoptosis. These results may be useful in further experiments involving endocrine regulation of oocytes in order to better understand the mechanism of the formation of fish oocytes.

INTRODUCTION

OOGENESIS AS A CONSERVED PROCESS IN THE EVOLUTION OF VERTEBRATES

The basic principles of oogenesis and their regulation seem to be evolutionary conserved among vertebrates (Urbatzka et al., 2011). From a comparative point of view, the endocrine and paracrine regulation of gametogenesis, the major developmental steps as well as the major player in this process have many in common across the species. Oocytes mature as follicle-enclosed oocyte complexes and grow arrested at the prophase of the first meiotic division, which re-initiation is an important step for achieving final oocyte maturation competence. The basic functioning of the reproductive axis– the hypothalamus-pituitary-gonad axis (HPG) - is similar in all vertebrates (Kah and Dufour, 2011; Urbatzka et al., 2011) albeit the phylogenetic distance between fishes and mammals. The action of neurotransmitters and neuropeptides on the hypothalamus leads to the release of gonadotropin releasing hormone (GnRH), which act on the pituitary to secrete gonadotropins (GTH) into the blood circulations that themselves exert several functions on the gonads. Especially due to these and other similarities, fishes are used as classical models to study the regulation of reproductive processes in vertebrates.

Also in lower vertebrates, oocytes grow within ovarian follicles arrested at the first meiotic prophase, but in contrast to mammals yolk proteins are accumulated into the cytoplasm during oocyte development (Nagahama and Yamashita, 2008). Furthermore, fishes possess a high variability regarding their different reproductive strategies. They can be characterized by different ovarian development: a) synchronous, where oocytes are formed and ovulated at the same time; b) group-synchronous, where two different stages are visible in the gonad; c) asynchronous, where oocytes of all stages are represented in the ovary at the same time (Wallace and Selman, 1981). These strategies mentioned above have evolved along the time and may be one of the reasons for the evolutionary success of the teleost group. Teleost represents the largest (about 30,000 species) and the best studied group of fishes and among vertebrates (Jalabert, 2005). Due to the huge diversity of sexual phenotypes and reproduction strategies, this group, that includes the zebrafish (*Danio rerio*) is very well suited to study mechanisms of reproduction (Paul-Prasanth et al., 2011).

MORPHOLOGICAL CHANGES DURING OOCYTE DEVELOPMENT

Although the egg formation and spawning may differ between fish species, the major steps in the oocyte development are similar in teleosts (Lubzens et al., 2010).

In the following is described a characterization of zebrafish oogenesis.

Primordial germ cells (PGC) are formed during embryonic development (Devlin and Nagahama, 2002) and play a crucial role in the development of gonads (Braat et al., 1999). These cells are distinguishable by their small size (20 μm) and their spherical form with a large nucleus, aggregated in nests and bordered by pre-follicle cells (Selman et al., 1993; Yoshizaki et al., 2002). PGC develop into oogonia, and then into oocytes, where five different stages of development are described in the literature (Wallace and Selman, 1981; Wallace and Selman, 1990; Selman et al., 1991; Selman et al., 1993; Clelland and Peng, 2009).

Stage I

Stage I is the so called “primary growth stage” (Wallace and Selman, 1981; Selman et al., 1993) and can be divided in two different sub-stages: the pre-follicle phase (stage Ia) and the follicle phase (stage Ib) (Selman et al., 1993). In stage Ia, the chromatin is highly condensed in the nucleus containing a single and a large basophilic nucleolus (Wallace and Selman, 1981). The oocytes are organized in a sort of nests and in similar phases of the prophase with a diameter between 7 and 20 μm . In stage Ib, several perinucleous are formed (Yamamoto, 1956), which migrate to the periphery of the germinal vesicle (GV). The chromosomes begin to decondense in the diplotene, when the development is arrested (Selman et al., 1993). The oocytes grow from 20 to 140 μm during this stage. Furthermore, a thin connective tissue starts to surround the nascent oocyte, which later will turn into thecal cells (Wallace and Selman, 1990). The differentiation of mesenchymal cells into thecal cells is forming an outer layer of follicle cells separated from the granulosa layer, which is derived by an epithelial compartment of the oocyte, the basal lamina (Abraham et al., 1984; Wallace and Selman, 1990; Lubzens et al., 2010). At this stage, the vitelline envelope (with a thickness of about 15 μm) begins to form in the perivitelline space (zona radiata) that is constituted by two acellular layers. For the development of these layers, zona pellucida (zp) glycoproteins are incorporated in vesicles, which fuse with the oocyte plasma membrane (Modig et al., 2007). Later, a second layer called zona radiata interna is secreted from the oocyte displacing the zona radiata externa for the

granulosa cells (Lubzens et al., 2010). In parallel to the formation of the zp, microvilli are emerge mostly from the oocyte to the follicular layers (Selman et al., 1993) and are fundamental for the communication between cells.

Stage II

The second oocyte stage is named “cortical alveoli stage” due to the appearance of CA, which lead to the enlargement of the oocyte (Wallace and Selman, 1990; Selman et al., 1993; Lubzens et al., 2010). The follicle usually reaches sizes between 140 and 340 μm (Selman et al., 1993), and the GV become irregular (GV reticulation). At the end of this stage, CA occupies almost all cytoplasm (Wallace and Selman, 1990; Selman et al., 1991; Selman et al., 1993) and lysosome - like vesicles appear to participate in the formation of yolk (Begovac and Wallace, 1988). Initially, CA were wrongly defined as yolk bodies (Wallace and Selman, 1981), however they can be stained for proteins and carbohydrates (Wallace and Selman, 1981; Lubzens et al., 2010), which are endogenously produced with the involvement of the ER and the Golgi apparatus (Wallace and Selman, 1990). After fertilization, the content of CA is released to the egg surface to prevent polyspermy and to induce the restructuring of proteins from the egg envelope to form the chorion (Wallace and Selman, 1990; Selman et al., 1993; Lubzens et al., 2010). Furthermore, three different layers in the vitelline envelope (Abraham et al., 1984) are formed, the zona radiata externa, zona radiata interna 1 and zona radiata interna 2 (Selman et al., 1993), which is crossed by microvilli.

Stage III

Stage III of the oocyte development is also called the “vitellogenic stage”, because of the strong incorporation of the hepatic protein vitellogenin (Vtg) (Wallace and Selman, 1981). The cortical vesicles are progressively distributed to the periphery of the oocyte, while the yolk bodies are accumulated in the centre of the cell (Selman et al., 1993). Yolk bodies can reach a maximum of 40 μm as a result of the Vtg uptake. Vtg reaches the oocyte by passing the thecal capillaries to the granulosa layer and then the pore channels of the zona radiata at the oocyte surface. Following this, Vtg is sequestered by specific receptors that promote the endocytosis of this protein in clathrin-coated vesicles that fuse with lysosomes and form multivesicular bodies (MVB) (Wallace and Selman, 1990). Here, Vtg is metabolized and incorporated into yolk bodies (Guimarães and Quagio-Grassiotto, 2005). In addition, other proteins, lipids, vitamins or carbohydrates are uptake by the oocytes (Lubzens et al., 2010), which serve as an indispensable reserve for the nutrition of the embryos (Brooks et al., 1997; Bobe and Labbé, 2010). At stage III, the follicle

diameter ranges from 340 to 690 μm and the vitelline envelope gets progressively thinner (Abraham et al., 1984), while the follicle cells enlarge and increase. The GV becomes less evident and begins to move to the periphery.

Stage IV

In the maturational stage IV, meiosis is first reinitiated, but then the oocytes get arrested again at the metaphase until fertilization, which is the signal to terminate the meiosis. The oocytes are able to respond to maturation-inducing steroids and in consequence the GV moves towards the exterior part of the oocyte (GVBD- germinal vesicle break down) and the first polar body is extruded (Nagahama, 1994). During this stage, the follicle diameter ranges between 690 and 730 μm and the volume of oocytes of zebrafish increase about 10-15%, depending on the osmolarity and the composition of the medium surrounding the cells (Selman et al., 1993). Before ovulation, the follicle cells and their pore canals retract from the oocyte, while the microvilli continue to develop from the oocyte to the vitelline envelope even after ovulation (Selman et al., 1993).

Stage V

In the final stage V, the complete developed oocyte has a diameter between 730 – 750 μm and here, the oocytes do not have the follicles cells that used to surround them. The vitelline envelope (about 3 μm thick) is still tripartite, perforated by a micropyle and yolk bodies are visible within the oocytes (Selman et al., 1993).

ENDOCRINE REGULATION OF OOCYTE DEVELOPMENT

Oogenesis is regulated at a higher level through the HPG axis by the secretion of GTH (Feist and Schreck, 1996; Clelland and Peng, 2009). GTH regulate the ovarian development and play an important role during growth, vitellogenesis and maturation of the oocytes (Nagahama, 1994; Rocha and Reis-Henriques, 1998; Rocha and Rocha, 2006; Urbatzka et al., 2011). Two types of GTH are known: GTH-I and GTH-II, which are similar in function and structure to the FSH and LH, respectively, of mammals (Rocha and Rocha, 2006). GTH stimulate the production of testosterone and 17 α -hydroxyprogesterone by the follicular cells of theca. Later on, and depending on the maturative stage of the gonad, testosterone is then converted into E₂ and 17 α -hydroxyprogesterone into DHP at the granulosa layer (Rocha and Rocha, 2006). A high correlation of FSH with E₂ and of LH with DHP was verified in salmonids (Swanson, 1991), tilapia and sea bass (Rocha and Reis-Henriques, 1996; Rocha and Reis-

Henriques, 1998; Rocha and Reis-Henriques, 2000; Rocha and Rocha, 2006). It is thought that FSH is primarily involved in the regulation of the oocyte growth, while LH acts on the maturation of oocytes. Thereby, FSH stimulates the production of E₂ in follicular layers in the later stages of oocyte development, especially at the vitellogenic stage III. E₂ is secreted into the blood circulation and induces the liver to produce Vtg. In turn, Vtg is accumulated in the oocytes and stored as yolk globules, a process that is also promoted by FSH (Tyler et al., 1991). GTH also regulate the expression of activins (Pang and Ge, 2002c), which levels increase during vitellogenesis and are involved in follicle growth and maturation (Pang and Ge, 2002b).

A two-stage model of maturation of the oocyte has been proposed (Patiño et al., 2001). In a first step, LH has a priming effect on the oocyte, which “prepares” it to the maturational effects of the final maturation where (Nagahama et al., 1993; Patiño et al., 2001; Yoshizaki et al., 2001; Patiño and Sullivan, 2002; Nagahama and Yamashita, 2008; Clelland and Peng, 2009) MIH receptors increase on the oocytes membrane and in consequence, the oocytes become competent to mature (Pang and Ge, 2002b; Pang and Ge, 2002c). Then, in the second step, LH stimulates the production of MIH and consequently the induction of GVBD (Patiño et al., 2001) by the activation of the maturation-promoting factor (MPF) (Nagahama and Yamashita, 2008) and by the interaction via specific MIH-receptors on the membrane of the oocyte (Zhu et al., 2003; Clelland and Peng, 2009). This model has been proven in at least ten teleosts and one amphibian species (Patiño et al., 2001). However, many other proteins and factors seem to be involved in the process of maturation as activin, serotonin, follistatin (Pang and Ge, 2002c) epidermal growth factor (EGF) or transforming growth factor β (TGF- β) (Pang and Ge, 2002a). Also insulin-like growth factor I (IGF-I) (Nelson and Van Der Kraak, 2010) and in some cases insulin-like growth factor II (IGF-II) (Bobe et al., 2003) seem to have an important role in promoting the oocyte maturation competence (OMC) (Bobe et al., 2003; Campbell et al., 2006; Nelson and Van Der Kraak, 2010).

Some morphological events occur just before the ovulation. Microvillis between the oocyte and granulosa are broken by the action of enzymes that digest the follicular layer and lead to ovulation (Clelland and Peng, 2009). In medaka, those enzymes have been identified as gelatinase A and metalloproteinases 1 and 2 (Ogiwara et al., 2005). DHP have a great importance not only on the maturation of the oocyte, but also on the ovulation according to the localization of the DHP receptors in the follicle (Nagahama and Yamashita, 2008). During maturation, DHP acts over membrane bound receptors, whereas in ovulation it acts via nuclear progesterone receptors. These nuclear receptors

occur only after the action of DHP during maturation (Liu et al., 2005) and thus, the hormone act via a different pathway during the process of ovulation.

Sometimes after ovulation, the phenomenon of hydration of oocytes occurs and despite the fact that hydration is more common in marine species (Cerdà et al., 2007), it is also found in some fresh water animals (Babiker and Ibrahim, 1979a; Milla et al., 2006). In marine pelagophil fish, the final volume of the oocyte can reach 67-75% due to the water uptake by aquaporins (Cerdà et al., 2007), whereas in benthophiles it can augment to 85% (Lubzens et al., 2010). Zebrafish spawn non-hydrated eggs (Lubzens et al., 2010) and the increase of 10-15% of volume mentioned by Selman et al (1993) is not considered significant.

IMPORTANCE OF THE EARLY STAGES

Despite of the amount of information that has emerged in the past decades about the oocyte development, there is a lack of information about the early stages of oocytes. Very little is known about what triggers the recruitment of primordial germ cells and their fate and about the molecular mechanisms directing the primary growth phase (Urbatzka et al., 2011). The primary growth phase is regarded as fundamental for the development of the oocytes as well as for the embryos since important processes takes place during the development of the early oocyte steps comprising amongst others the formation of the follicle cells, the inclusion of maternal RNA and the initiation of the synthesis of lipid droplets.

Follicle cells

Follicle cells are common in vertebrates (Gosden et al., 1997; Yamamoto et al., 2002). The first monolayer of follicle cells is composed of granulosa cells that are irregular in shape and rich in lipid droplets (Pang and Ge, 2002c). Above these cells grows a layer of thecal cells (Clelland and Peng, 2009; Clelland and Kelly, 2010), which are elongated and posses a large nuclei. Follicular cells produce sexual steroids, which in turn have an essential role in the oocyte growth and maturation (Nagahama and Yamashita, 2008). Moreover, these cells are endowed of several specific hormone receptors (Pang and Ge, 2002c; Zhu et al., 2003) and produces further factors that are involved in the oocyte development. The communication of follicular layers occurs between the follicular cells and between follicular cells and the oocyte. Granulosa cells project microfilaments through the zp of the oocyte, where gap junctions (GJ) form that allow the sharing of small

molecules (<1kDa) between granulosa cells and the oocyte (Grazul-Bilska et al., 1997; Kidder and Mhawi, 2002). GJ are structured by different connexin proteins that are organized into connexons, which form intercellular channel, when a connexon of one cell connects to a connexon of other cell (Grazul-Bilska et al., 1997; Kidder and Mhawi, 2002). In addition to the important impact that follicles cells possess on the development of oocytes, many studies suggest a bidirectional communication between the follicular cells and the oocytes, which involves an active role of the oocyte in the regulation of the surrounding cells (Gosden et al., 1997).

Lipid droplets

In some fish species as bluefin tuna (*Thunnus thynnus*), the incorporation of lipid droplets has such an importance that a transitional stage was considered, termed “lipid stage” (Abascal and Medina, 2005). Lipid droplets are important for the nutrition of the future embryo and for the egg shell formation. Studies demonstrated that the presence of fatty acids in the culture medium triggers the formation of lipid droplets (van Meer, 2001; Pol et al., 2004; Martin and Parton, 2005). These structures are also abundant in steroidogenic cells in the adrenal cortex, ovary and testis (Martin and Parton, 2005). Their biogenesis is unclear, but associated to several organelles as ER, especially in a specific subdomain of the ER membrane (Denis, 2001), and mitochondria (Żelazowska and Kilarski, 2009).

Incorporation of maternal RNA in early stages

Some studies reveal a crucial role of maternal genes for embryonic development, especially for the regulation of animal-vegetal polarity, egg activation, cleavage development, body plan formation and tissue morphogenesis, but also in the germ cell development (Braat et al., 1999; Slanchev et al., 2005; Abrams and Mullins, 2009). Almost all studies used zebrafish mutants for specific genes to analyze and characterize the function of the maternal genes that are generated during oogenesis, supplied to the egg, and already present in the early stages of oocyte development (Abrams and Mullins, 2009).

STATE OF KNOWLEDGE OF ENDOCRINE REGULATION OF EARLY STAGES

Primary growth is believed to be GTH-independent (Wallace and Selman, 1981), because in hypophysectomised teleosts there was no inhibition of the primary growth oocytes (Khoo, 1979; Billard, 1992; Clelland and Peng, 2009). However, studies in salmonids revealed that FSH may be involved in the recruitment of primary oocytes,

because of its increase during ovulation (Prat et al., 1996; Santos et al., 2001), which precedes directly the initiation of a new cycle of oocyte development. Moreover, transcripts of genes encoding the β -subunits of GTH (FSH β and LH β) and their respective proteins were found in the primary oocytes of gilthead seabream (*Sparus aurata*) (Wong and Zohar, 2004). These last results suggest that GTH may have a role in the initiation and folliculogenesis of fish oocytes as it is known from mice (Wang and Greenwald, 1993). Furthermore, the increase of FSH levels in plasma and pituitary, plasma E₂ and IGF-I levels were associated to the CA development, which potentially interact with the body growth of the organism (Kwok et al., 2005; Campbell et al., 2006; Lokman et al., 2007). Nonetheless, more studies are needed to understand the action of GTH in the transition from stage I to stage II (Lubzens et al., 2010).

Sexual steroids are interesting candidates, which may be involved in the development of early oocytes. However, they are mainly known as direct effectors of oocyte development in the later stages. In the following, some of the known effects of hormones on oocyte development are discussed with special focus on the early stages.

E₂ is the main female sex steroid which is involved in sexual differentiation (Simpson et al., 2005), vitellogenesis and oocyte maturation (Lubzens et al., 2010). As mentioned before, it has a particular action on the formation of CA (Campbell et al., 2006), and seems involved on the formation of tight junctions (Clelland and Kelly, 2010). An impact on the DNA proliferation was shown on the oocyte early diplotene and on oogonial proliferation (Miura et al., 2007).

11KT, the oxidized form of testosterone, is the major male sexual steroid in fish, because of its importance in male differentiation. Because androgens may have an important effect on previtellogenic oocytes and on oocyte growth (Kortner et al., 2008) they may also be responsible for increasing the size of perinuclear oocytes and for the increase of lipid accumulation inside the oocyte in the presence of triglycerides in the culture medium (Lokman et al., 2007).

DHP induces the development of progesterone receptors on the surface of the oocyte in the maturational stages (Zhu et al., 2003). Once achieving the progesterone receptors, DHP triggers a biochemical cascade of actions in the cell that leads to the final maturation. In the early stages, recent studies identified high levels of membrane bound progestin receptors (mPr). Furthermore, hsd3b and hsd20b, enzymes responsible for the production of progesterones, are present in the early stages of oocyte development

(Clelland and Peng, 2009), which lead us to believe that DHP has also an action on the primary steps of oogenesis. Moreover, this steroid may be the indicator of the first meiotic division in the oogenesis in carp and huchen (Miura et al., 2007).

Stress hormones may interfere with the normal development of oocytes, but also as mediators of a crosstalk of endocrine systems. It is well known that stress negatively interferes with reproduction, but mechanisms are less clear. Besides, there is little information about the effect that these hormones might have on early oocytes stages. Cortisol, a glucocorticoid that is released into the blood circulation in stress conditions and at low glucose levels (Mommsen et al., 1999), is involved in the ovulation in some fishes (Hirose et al., 1974; Babiker and Ibrahim, 1979a; Babiker and Ibrahim, 1979b; Bry, 1985) and enhances the action of some hormones that induces GVBD. The presence of mRNA levels of 11 β -hydroxylase, an enzyme involved in the synthesis of cortisol (Mommsen et al., 1999), in the ovary of rainbow trout during oocyte maturation (stages III - IV) (Bobe et al., 2003; Bobe et al., 2004) is an indication that cortisol may have an action during the development of the oocyte.

A recent work describes the presence of mRNA for melanocortin 2 receptor (MC2R), the specific receptor for ACTH in fish gonads (Aluru and Vijayan, 2008), including zebrafish (Alsop et al., 2009). ACTH is the hormone secreted by the pituitary gland that is responsible in mediating a stress response. This principal regulator of corticosteroid synthesis acts on the interrenal cells of the head kidney and stimulates the cortisol release into the blood during a stress response (Fuzzen et al., 2011). Co-administration of human chorionic gonadotropin (hCG) with ACTH results in the suppression of the hCG induced biosynthesis of E₂ (Alsop et al., 2009). These results suggest that long-term stress conditions lead to the excretion of ACTH that might result in dysfunctional reproduction.

ZEBRAFISH AS MODEL

Many endocrine studies use the zebrafish (*Danio rerio*) as model and many of those studies were referenced before. The main advantages of using this fish are the easy maintenance and control of reproduction in laboratory (Westerfield, 1995a; Westerfield, 1995b; Brand et al., 2002), the transparency of the eggs for studies of developmental biology (Lele and Krone, 1996), neurophysiology, toxicology (Carvan III et al., 2005) or biomedicine (Zon and Peterson, 2005) and for the well known, sequenced and explored genome (Spitsbergen and Kent, 2003).

For this work, zebrafish has also the advantage of being an asynchronous spawner, which means that all different oocyte stages are present simultaneously in the ovary. A

special feature of the zebrafish reproductive biology is that zebrafish is a protogynous hermaphrodite (Rocha and Rocha, 2006). All individuals develop first ovary-like gonads ("juvenile ovary") (Takahashi, 1977), which at 5 to 7 weeks post-hatching some of the animals differentiate into adult, mature gonads, whereas others undergo sexual transition and form differentiated testis (Orban et al., 2009). This phenomenon seems to be genetically controlled, however it might be influenced by environmental factors (Uchida et al., 2004; Slanchev et al., 2005). During this process of sex reversal, the degeneration of oocytes in the gonads of some zebrafish juveniles is caused by apoptosis (programmed cell death) (Uchida et al., 2002).

Studies in zebrafish oocytes revealed that disappearance of apoptotic oocytes from gonads was followed by differentiation of spermatogonia, which may indicate that apoptosis of those oocytes is male-specific and a mechanism to promote testicular differentiation (Uchida et al., 2002; Orban et al., 2009). This phenomenon was observed between 21 and 25 days post hatching, when oocytes were in the early diplotene suggesting that a proapoptotic signalling pathway to sex-determination is present in oocytes and may be stimulated by changes in hormones or sex-determining genes (Uchida et al., 2002; Wang et al., 2007).

The follicle cells also change their appearance during the process of sex reversal undergoing masculinisation: granulosa and thecal cells become Sertoli and Leydig cells, respectively (Guigon et al., 2005). The somatic cells of the stroma divide along the peripheral region of the gonad. The gonial germ cells become separated by stroma cells and then start to multiply originating seminal lobules (Orban et al., 2009). Males become completely distinguishable from females at about 35 dph (days post hatching) (Takahashi, 1977).

AIMS OF THE STUDY

As outlined before, the early stages of oocyte development are fundamental for the development of the oocytes and for the later embryo, since many important processes take place or initiate during these stages. However, little is known so far on the physiological and molecular mechanisms governing the regulation of the oocyte development in the early stages, but indications exist that hormonal signalling and the action of different peptide factors may be involved in these processes.

On one hand, the principal objectives of this work were to analyze if a certain set of hormones plays a role in the maturation of the early stages of oocytes or in the formation of their surrounding follicular cells. On the other hand, we aimed to investigate if hormones may have the potential to induce apoptosis in different stages of oocytes, which could be an endocrine/paracrine signal during the process of sex-reversal.

At first, an analysis of follicle-enclosed oocyte cultures was performed *in vitro* under serum-free culture conditions followed by a detailed characterization of the morphological alterations of oocytes along the time using LM and TEM. With this approach, it was intended to verify and improve the *in vitro* oocyte culture as well as to determine the maximum culture time.

Then, zebrafish oocytes were exposed *in vitro* to a set of endogenous hormones and cAMP in order to evaluate their role in early stages of oocyte development. The sexual steroids were E₂, 11KT, DHP. Beyond this, the role of two stress hormones as Cort and ACTH were also studied. Hereafter, when referring to exposure to hormones, the second messenger cAMP is included, simulating the action of GTH.

Histological techniques were used to analyze potential alterations of the hormonal exposure on general aspects of the morphology of the oocytes, while TEM allowed the observation of ultrastructural details not only of the oocyte but also from follicular layers. A stereological approach was used to reveal potential alterations on the granulosa layer, especially alterations concerning the cell proliferation. Immunohistochemistry (IHC) was used with a specific antibody for E-cadherin, which is involved in the formation of the heterotypic adherens junctions (Cerdà et al., 1999). With this approach, it was intended to find out if the development of this type of junctions can be stimulated by hormones.

Finally, the comet-assay (Ostling and Johanson, 1984; Fairbairn et al., 1995) was performed with oocytes at stage I and II in response to the same set of hormones to detect the induction of DNA breaks, single and double stranded breaks (Liao et al., 2009), which are signs of cellular apoptosis. These last results could be related to the hormonal-induced apoptosis that occurs in the gonads of vertebrates, but especially to the sex-reversal characteristics of zebrafish.

In vitro modeling of the oocyte development in zebrafish (*Danio rerio*): the role of hormones in maturation, apoptosis and sex-reversal of oocytes at different development stages

METHODS

ANIMAL MAINTENANCE

The zebrafish were produced under laboratory conditions. The progenitors were kept at 26°C in 30L tanks connected to a recirculation system with a 14:10 light/dark photoperiod cycle. Males and females were separated with a net and fed twice a day with dry flake food (TetraMin, Tetra, Germany) and once a day with 48h hatched brine shrimp (*Artemia salina* nauplii) (Ocean Nutrition, Belgium). After a week, the animals were transferred into a clean tank with the ratio of 4 males to 8 females, about 1 to 2 h before the end of light period. In the next light cycle, the embryos were collected and cleaned with distillate water at 26°C and then they were kept at 28°C. After 4 days post hatching (dph) larvae were fed with 24h hatched brine shrimp twice a day. At 21 dph animals were transferred to 30L aquaria at 26°C and started to be fed with dry food (Westerfield, 1995b).

ISOLATION OF OVARIAN FOLLICLES AND *IN VITRO* CULTURE

Mature, six months old females were anesthetized with 0.01% (v/v) 2-phenoxyethanol (Sigma, Switzerland) for 5 min and decapitated before dissection. The ovaries were removed and cleaned in a phosphate-buffered saline (PBS) solution with 1% penicillin and streptomycin (Sigma-Aldrich, USA) and 0.1% amphotericin B (Sigma-Aldrich, USA). The oocyte cultures were performed in two different ways: with fragments of ovaries sliced into pieces of 1-2 mm diameter and with isolated oocytes, where the oocytes were carefully separated by needles, smooth pipetting and a with gentle agitation in EDTA 0.1M (Cerdà et al., 1999) for two rounds of 15 min. Then the follicles were sorted by size (Pang and Ge, 2002b) and placed in 24-well plates with 500 µL of 75% and 90% medium Leibovitz L-15 (Sigma Aldrich, USA) (Seki et al., 2008) with 1% penicillin and streptomycin (Sigma-Aldrich, USA) and 0.1% amphotericin B (Sigma-Aldrich, USA). Plates were incubated at 26°C.

Oocytes, separated by stage and placed in different concentration medium, were measured under a stereomicroscope (Olympus, SZX10) coupled to a SZX2- ILLT led transmitted light illuminated stand and to a digital camera (Olympus, DP21).

VIABILITY TEST

To test the viability of the isolated oocytes over time a 0.1% trypan blue staining test and histology procedures were performed. Oocytes that stained blue were considered

unviable, while oocytes that maintained their colour were considered viable. The oocytes were cultured for 6 days and the viability test was performed every 2 days. In parallel, some of the oocytes were chosen from the culture every 2 days for histological processing (paraffin sections) and observation as described later. The maximum of oocytes were counted for each sectioning and classified as viable and unviable according to their apparent integrity.

LIGHT MICROSCOPY

Oocyte cultures were regularly observed under a contrast phase Olympus CKX41 inverted microscope for a first impression of the viability of the cultures and possible contaminations.

CONTAMINATION CONTROL

Suspected observations of contamination of the culture were tested by performing cytopins of the culture medium. Approximately 100µL of the medium was centrifugated in a StatSpin CytoFuge 2, for 6 min at 2148 rpm. The slides were air-dried at room temperature and then Romanovsky staining were used (1 min in methanol, 2 and 3 min in hemacolor solutions 2 and 3, respectively (Merck, Germany)). After drying, the coloured slides were mounted in DPX mounting media, observed with an Olympus CX21 light microscope and photographed in an Olympus DP21 digital camera.

PARAFFIN SECTIONS

Oocytes (separated by stage or fragments) were removed from the culture and fixed in 10% buffered formalin for 24 h. They were then embedded in Histogel™ (Thermo Scientific, Germany) previously heated to 60 °C. Stages I and II were centrifugated for 2 min at 2000 rpm and stages III and IV were kept at 37 °C until sedimentation to the bottom of the tube. After cooling, samples were stored in 70% ethanol at room temperature before being processed in an automatic tissue processor (Leica TP 1020, Germany) following a routine procedure (1 h in each bath): 70% ethanol, 90% ethanol; 96% ethanol; 99,9% ethanol (dehydration) and mixture of ethanol 1:1 xylene, xylene (clarification); paraffin (56 °C) (impregnation). Afterwards, the samples were embedded in Histosec paraffin wax. Sections with 5 µm were cut with a Leica 2155 microtome, mounted in slides treated with 3-aminopropyltriethoxysilane (Apes) and left overnight in the oven at 37 °C. Slides were deparaffinised and rehydrated. For LM, slides were stained with hematoxylin

Gill II (Merck, Germany) for 4 min and eosin Y 1% (yellowish) for 1 min (Merck, Germany), dehydrated, cleared in xylene or with the specific stains described below. At last, slides were mounted in DPX mounting media and observed in an Olympus CX21 light microscope and photographed with an Olympus DP21 digital camera.

GOLDNER TRICHROME STAINING (GOLDNER, 1938)

This staining was already used in oocytes of a roach, *Rutilus rutilus*, in a previous study (Trubiroha et al., 2011). The CA containing carbohydrates stain of blue whereas yolk bodies stains of red. First staining was with hematoxylin Gill II solution (Merck, Germany) for 4 min followed by washing in running tap water for 5 min. The Masson's 1% acid fuchsin – 1% xylydine ponceau mixture (1:2) for 5 min was then used before rinsing with 1% acetic acid. Slides were treated with phosphomolybdic acid for 15 min, then stained with 0.5% Orange G for 5 min and rinsed again with 1% acetic acid. Finally, slides were stained with 0.2% Light green for 15 min, washed with 1% acetic acid, air-dried and mounted in DPX mounting media after a quick passage in absolute ethanol and xylene.

AZOCARMINE & ANILINE STAINING – AZAN (ROMEIS, 1948)

This staining can be used for general histology. Usually, nuclei, erythrocytes, fibrin, fibrinoid, acidophilic cytoplasm, epithelial hyaline are stained of red by azocarmine. Collagen fibers, basophilic cytoplasm and mucus are counterstained with blue or orange by aniline blue or orange G, respectively (Hasumi et al., 1990).

The protocol described above was based in Hasumi et al.(1990) work which was in turn an adaptation from the original (Romeis, 1948). The slides were treated with a mordent solution (1:1 of 10% potassium dichromate and 10% trichloroacetic acid) and rinsed with distilled water for 5 min. The first dye was 0.5% Orange G with 1% acetic acid for 10 min followed by 5 min in distilled water. After 20 min of 0.1% azocarmine B, the slides were washed in running tap water for 5 min, differentiated in a solution of 0.1% aniline in ethanol 96%, and a washed first with a solution of 0.1% acetic acid in ethanol 96% and then in running tap water for 2 min. They were then incubated in 5% phosphotungstic acid for 1 h 30 h. After washing in running tap water for 2 min, slides were finally stained with 0.25% aniline blue and 4% acetic acid for 15 min, dehydrated, cleared and mounted in DPX mounting media.

IMMUNOHISTOCHEMISTRY

Sections of 3 μm were cut with a Leica 2155 microtome, mounted on Apes-treated slides, and left overnight in the oven at 37 °C.

Slides were deparaffinized and rehydrated. High temperature antigen retrieval was performed in a microwave at 600W with pre-warmed Tris-EDTA buffer (10mM Tris Base, 1mM EDTA, pH 9.0 with 0.05% Tween 20) for 10 min. Endogenous avidin/biotin was blocked using a blocking kit (Zymed) according to the instructions of the manufacturer. Also, endogenous peroxidases were blocked by immersion in a 3% hydrogen peroxide solution in methanol for 10 min.

Before incubation with the primary antibody, tissue sections were covered with the blocking solution of the Histostain-Plus Bulk Kit Detection system (Invitrogen, USA) for 15 min. The slides were then incubated with the monoclonal mouse anti-human E-cadherin antibody (Clone NCH-38) (Dako, Denmark) diluted 1:50 in PBS for 1 h 30. After washing twice in PBS (5 min each), the other components of the above mentioned kit (biotinylated secondary antibody and HRP Streptavidin/biotin complex) were applied for 15 min each. Slides were rinsed twice in PBS between both steps and before chromogenic reaction.

The DAB substrate kit (Zytomed, Germany) was used for the reaction visualization prepared following the instructions of the manufacturer. A slight tissue counterstaining was made with hematoxylin solution modified according to Gill II (Merck, Germany).

Positive and negative controls were included in order to assess the specificity of the reaction. Positive controls consisted of sections of zebrafish intestine and fresh gonad, collected and processed under the same conditions of oocytes, while primary antibody was omitted for negative control and replaced with PBS.

Finally, slides were dehydrated, cleared and mounted with DPX synthetic mounting media, observed with an Olympus BX50 light microscope and photographed with an Olympus Camedia C-5050 digital camera.

ELECTRON MICROSCOPY

Fragments of the gonad were removed and fixed in 2.5% glutaraldehyde in sodium cacodylate-HCl buffer (0.1 M pH 7.2) for 1 hour at 4°C. Then fragments were rinsed twice (30 min each) in the same buffer. Post-fixation was performed with a mixture of 1%

osmium tetroxide and 1.5% of potassium ferrocyanide in 0.1 M sodium cacodylate-HCl buffer for 2h or only overnight 4°C. The dehydration was performed with a series of ethanol of crescent concentrations from 50% to 100% (30 min each) and two passages in propylene oxide (15 min). The impregnation was made with crescent mixtures of Epoxy resin with propylene oxide (1:3, 1:1, 3:1) one hour for each mixture and finally with just Epoxy resin for 1h. Before inclusion, fragments were placed at 60°C for 10 min, and inclusion was made in rubber moulds. After polymerization at 60°C (48h) the blocks are trimmed and cut with an ultramicrotome Leica Reichert Supernova. Semithin sections (1µm thick) were stained with a mixture (1:1) of 1% methylene and 1% azur II. The ultrathin sections were placed in a 200 mesh hexagonal copper grids and contrasted with uranyl citrate (20 min) and lead citrate (15 min) (Smith and Melnick, 1962; Reynolds, 1963).

The grids were observed and photographed with an electron microscope JEOL 100CXII.

The contingency table was constructed by representing in percentages the qualitative observations of oocytes of six fishes.

ALKALINE COMET ASSAY

Frosted microscope slides (Superfrost®, Germany) were covered with 1% normal melting point agarose (NMA) in PBS and dried overnight at room temperature (Belpaeme et al., 1998; Lacaze et al., 2010). Oocytes of stages I and II were collected in medium and then the medium was reduced to 20 µL. 80 µL of 1% low melting point agarose (LMA) was added and the mixture loaded on the slide (over the NMA layer) and covered with a coverslip. A third layer of LMA was added above and then the slides were lysed in freshly made alkaline solution (2.5 M NaCl; 100 mM Na₂EDTA; 10 mM Tris-HCl, pH 10; 1% Triton X-100; 10% DMSO) at 4°C overnight. After lysis, the DNA unwinding was performed by submersion of the slides in alkaline solution (1 mM EDTA and 300mM NaOH, pH 13) for 40 min. Thereafter electrophoresis was conducted at 20V and 300mA for 30 min in the same buffer under ice-cold conditions. Slides were neutralized three times for 5 min with a neutralization buffer (Tris-HCl pH 7.5) and then stained with 0.02% of ethidium bromide for 15 min. After cleaning the slides with ultrapure water, they were observed under UV light with an inverted microscope Olympus IX71 and photographed by an Olympus DP71 camera.

The freeware Comet Score™ (freeware from TriTek, Summerduck, Va, USA) was used for scoring the comets and the results were analysed for the “tail moment”. “Tail moment”

is the product of the percentage of DNA in the tail with the length of the comet. All of those values are automatically obtained by the software.

To validate the method, hydrogen peroxide (H₂O₂) was used in three different concentrations, 100 µM, 250 µM and 500 µM, since it is known to act as a genotoxic substance commonly used as positive control (Nahon et al., 2008; Liao et al., 2009).

IN VITRO EXPOSURE EXPERIMENT

The exposure was performed in 24 well-plates, and six substances were tested: 17β-estradiol (E₂), 11-ketotestosterone (11KT), 17,20β-dihydroxy-4-pregnen-3-one (DHP), dibutyryl cyclic adenosine monophosphate (cAMP), hydrocortisone (Cort) and adrenocorticotrophic hormone (ACTH). The hormones and cAMP were all from Sigma Aldrich, USA, while ACTH was from NHPP (National Hormone and Peptide Program, CA, USA). These substances were dissolved in 99% ethanol and placed in the 90% Leibovitz L-15 medium in a final concentration of 10⁻⁶ M for hormones and 0.1% of ethanol in the control. The exposure lasted for 2 days (48h). The medium was changed every 24 h of exposure.

The fragments of ovaries were forwarded after the exposure to routine histology processing described above including TEM. Isolated oocytes were used after the exposure to perform the comet assay in the conditions described above.

STEREOLOGY

Two stereological approaches concerning the granulosa layer, were determined with the stereological software Olympus CAST- Grid (version 1.5, Olympus) coupled to system consisted by a microscope (Olympus BX50, Tokyo, Japan) equipped with a x100 oil-immersion lens (Olympus Uplan, NA=1.35) and a matching condenser, a motorized stage (Prior, Fulbourn, UK) with a 1µm accuracy in the x-y axis and a CCD video camera (Sony, Tokyo, Japan) connected to a PC monitor of 17 inches (Sony, Tokyo, Japan). The final magnification at the monitor of x4750 allowed the visualization of the follicular layers and the nucleus of the granulosa cells. Random semithin sections of the fragments of gonad exposed to the different hormones were observed. Consecutive fields around the oocytes were selected.

The nuclear v_v (volume- weighted mean value, µm³) of granulosa cells was estimated using the point-sampled intercept (PSI) method (Gundersen and Jensen, 1985).

A grid with segments and test points is used and the intersection between the profile of the nucleus of granulosa cell that the point hits is measure l_0 . The estimation of the nuclear volume is $\pi/3 \cdot \bar{l}_0^3$. The mean of all the measurements is obtained by the formula $\bar{v}_v = \pi/3 \cdot \sum l_0^3$. Over 150 nuclei for each stage and treatment were counted except for oocytes of stage IIb exposed to ACTH where it was only possible to count 64 granulosa nuclei.

The ratio surface/volume S_v (also called surface density, μm^{-1}) of the granulosa cells was obtained assuming the “isotropic uniform random” of the oocyte sectioning using the formula $S_v = 2 \times \left(\frac{P_t}{L_t} \right) \times \left(\frac{\sum l_i}{\sum P_r} \right)$ (Gundersen et al., 1988) where P_t is the total number of points in the grid, L_t is the total length of all the segments of the grid, I_i is the total number of intersections of the segments with the limits of the cell layer and the P_r which is the total number of points that hit the granulosa layer. Only stage I and II were analysed and the stages were quantified for “surface density of granulosa layer”.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

For all data, first the normality of the data was tested by using a normality test (Shapiro-Wilk normality test). None of the working data (culture conditions, stereological data, comet assay) had a Gaussian distribution and therefore non-parametric tests were performed. If two experimental groups were present, differences between the groups were calculated using the Mann Whitney U-test. If more than two experimental groups were present, a Kruskal-Wallis test was performed followed by Dunn’s Multiple comparison post-hoc test. In all analyzed data, differences of $p < 0.05$ were regarded as statistically significant.

In vitro modeling of the oocyte development in zebrafish (*Danio rerio*): the role of hormones in maturation, apoptosis and sex-reversal of oocytes at different development stages

RESULTS

GENERAL CHARACTERIZATION OF OOCYTES STAGES

Oogonia

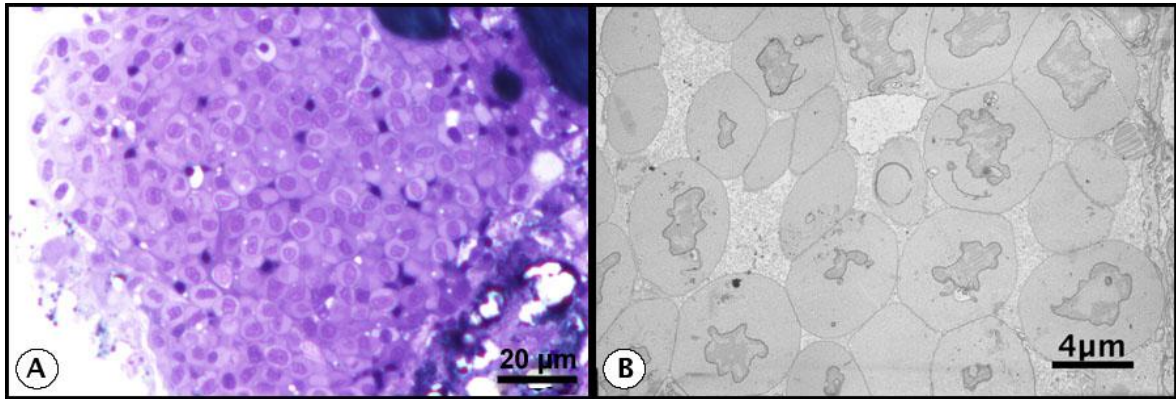


Fig. 1 Oogonia. A) LM. B) TEM.

Germ cells are clustered in kind of nests surrounded by an epithelial layer. The nucleus/cytoplasm ratio is high because the single nucleus occupies almost all the cell size (Fig. 1).

Folliculogenesis

The oocytes increase in size and are completely surrounded by a pre-follicular cell layer. The nucleus/cytoplasm ratio decreases. The GV develops from one single nucleus to several smaller electron-dense perinuclei that lie in the periphery of the GV. On the opposite side of the nuclear membrane appears frequently a slightly electron-dense granular material that is associated to clusters of mitochondria. With the development of the oocyte, the cytoplasm appears mottled with sER and Golgi apparatus. The mitochondria disperse from the clusters. The zp is absent in the beginning of stage I. Once the oocytes begin to develop the membrane projects microvilli through the outer follicular layer (Fig. 2). Later the zp begins to form and granulosa cells also begin to develop being flattened and having large mitochondria, sER and Golgi. Some rER is also visible, especially near the nucleus. The thecal cells are elongated with a large nucleus and some intracellular activity is visible by the presence of ER, mitochondria and small vesicles.

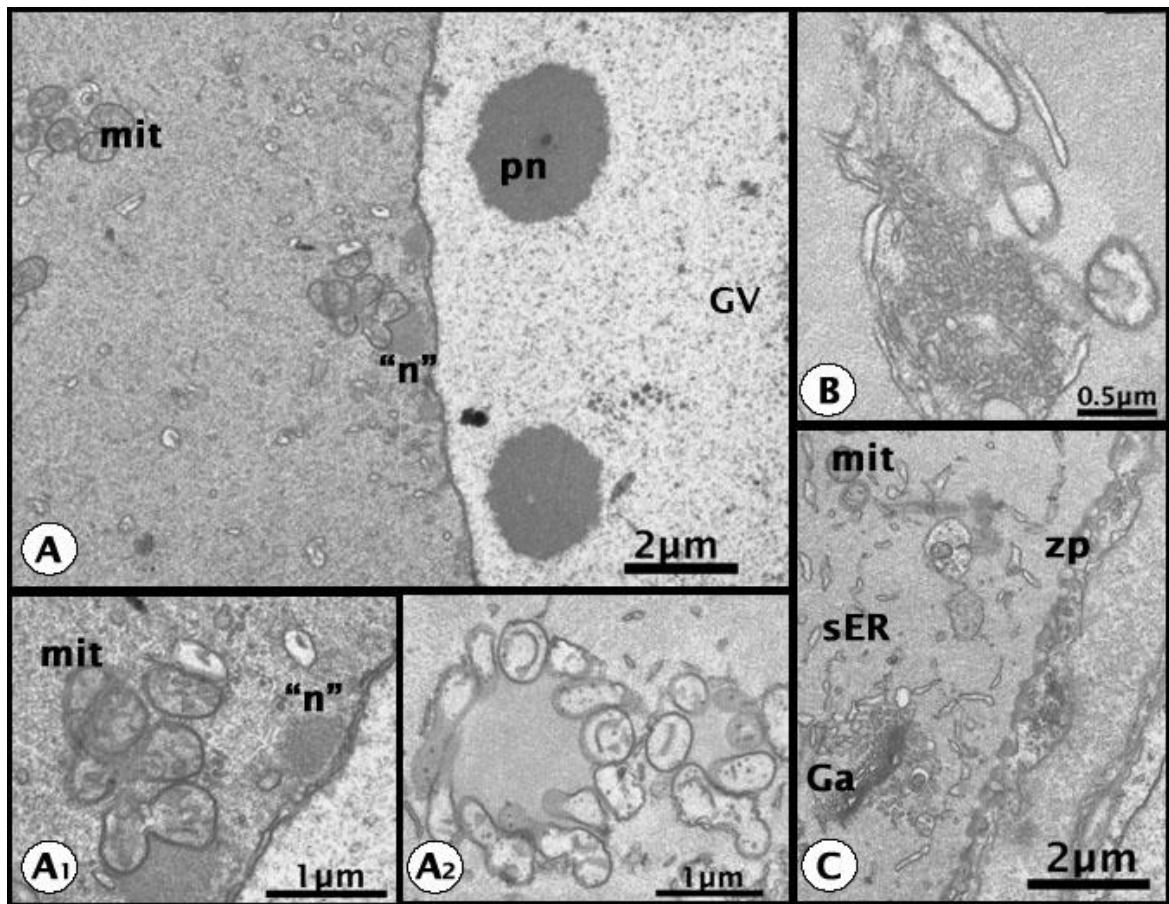


Fig. 2 Stage I (TEM). A) Inside of a stage I oocyte with germinal vesicle (GV) where and perinucleus (pn). Near the GV membrane there are “nuages” (“n”) clustered by mitochondria (mit). A₁) Mitochondria clustering nuage; A₂) Two “nuages” completely wrapped by mitochondria. B) A Balbiany body. C) A stage I membrane with a Golgi apparatus (Ga), smooth endoplasmic reticulum (sER) and mitochondria (mit) near the zona pellucida (zp) formation.

Primary growth

This phase is characterized by the appearance of CA. They appear initially in the periphery and later occupy the whole cytoplasm. Associated to CA, rER is present and ends in sER vesicles. Often clusters of organelles (*Balbiany bodies*) are visible in all cytoplasm as well as many mitochondria and free Golgi apparatus. The GV becomes reticulated. The microvilli at this stage crosses through the zp, but only reaches the granulosa cells and never pass the basal lamina. The follicular cells begin to enlarge and become rich in large and round mitochondria, rER cisternae and small vesicles. The thecal cells are more flattened than granulosa which exhibit more intracellular activity with defined organelles (Fig. 3).

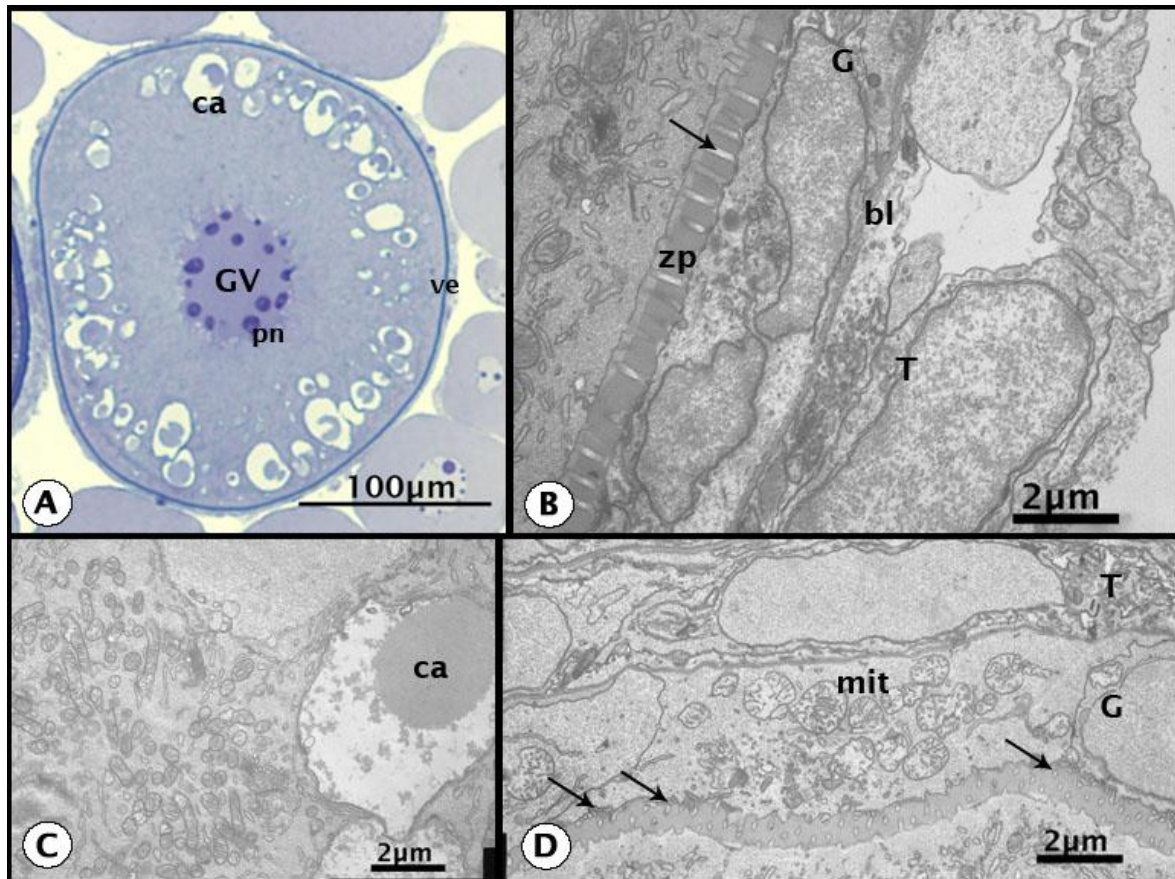


Fig. 3 Stage II. A) Oocyte at cortical stage (LM): germinal vesicle (GV) with several perinucleus (pn) inside. It is possible to see the development of the first cortical alveoli (CA) and the vitelline envelope (ve) (TEM). B) Follicular layers with zona pellucida (zp) and microvilli (arrows), basal lamina (bl) divides the granulosa layer (G) and thecal layer (T) (TEM). C) Inside of a cortical alveolar stage (TEM) with the cortical alveoli (ca). D) Periphery of an oocyte. Detail of the follicular cells with large and round mitochondrias (mit) and microvilli (arrows) across the zp reaching the granulosa layer (G) (TEM).

Secondary growth – vitellogenesis

With the uptake of Vtg, large electron-dense vesicles start to appear in the centre of the cell and spread until they occupy almost all the ooplasm. The yolk bodies present in this stage complicate the TEM procedure and the visualization under the electron microscope. The zp is large with a lot of microvilli and with some granular electron-dense material deposited on the outside (Fig. 4). The granulosa cells are now cuboidal, while the thecal cells are still elongated and the layer is from time to time separated by capillaries. Granulosa cells keep the large mitochondria, rER, sER, Golgi cisternae and small vesicles. Thecal cells have less apparent organelle activity showing however mitochondria, rER especially near the nucleus and Golgi. The detachment of this layer is very common due to the preparation.

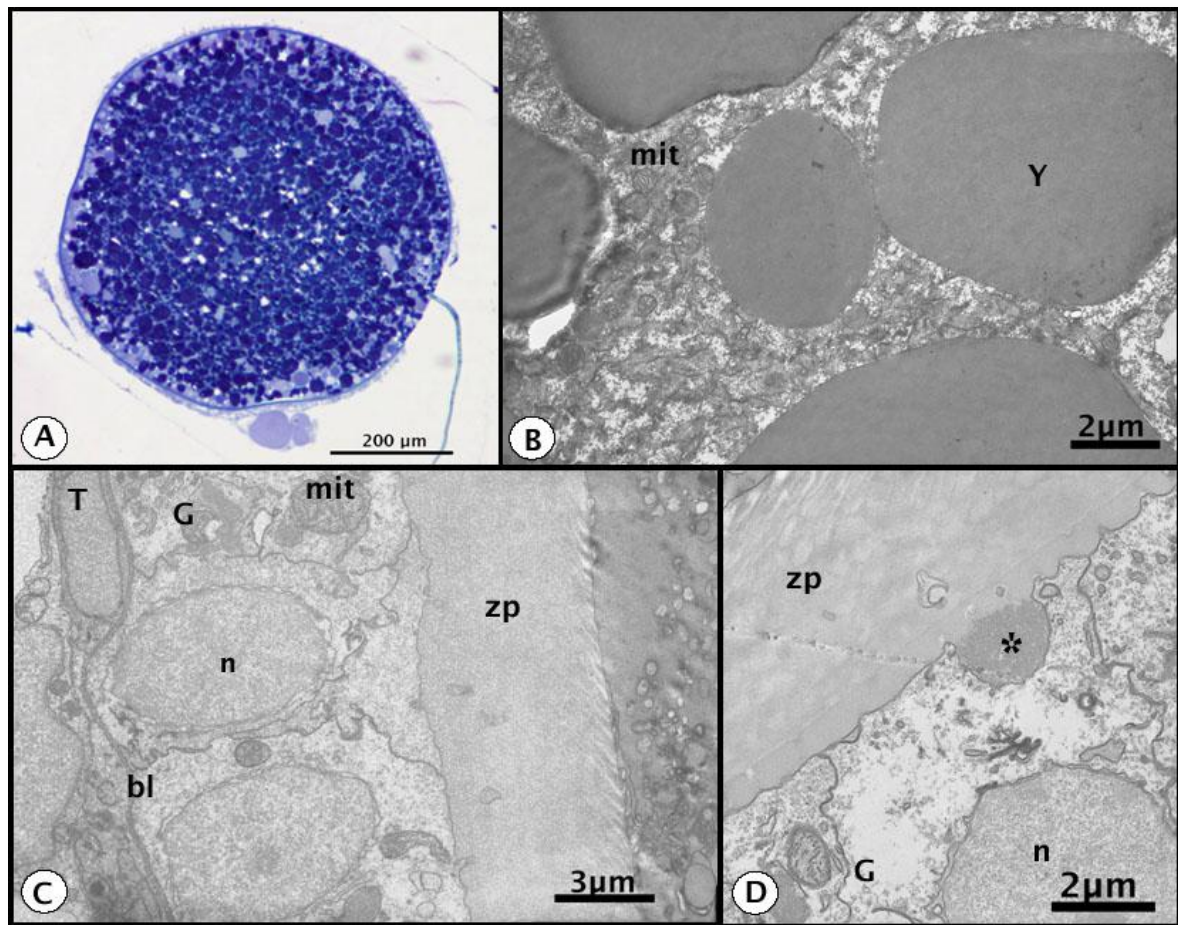


Fig. 4 Stage III. A) Vitellogenic stage (LM). B) Inside of a vitellogenic stage (TEM), yolk body (Y), mitochondria (mit). C) Periphery of a vitellogenic stage showing the developed follicular layers (TEM), nucleus (n), basal lamina (bl), zona pellucida (zp), granulosa cell (G), thecal cell (T). D) Detail of the zp and a granulosa cell with granular electrodense material (*) (TEM), zp (zp), granulosa (G), nucleus (n).

It is difficult to distinguish morphologically if an oocyte has undergone maturation. More advanced oocyte stages exhibit coalescence of the already large yolk globules (Fig. 5).

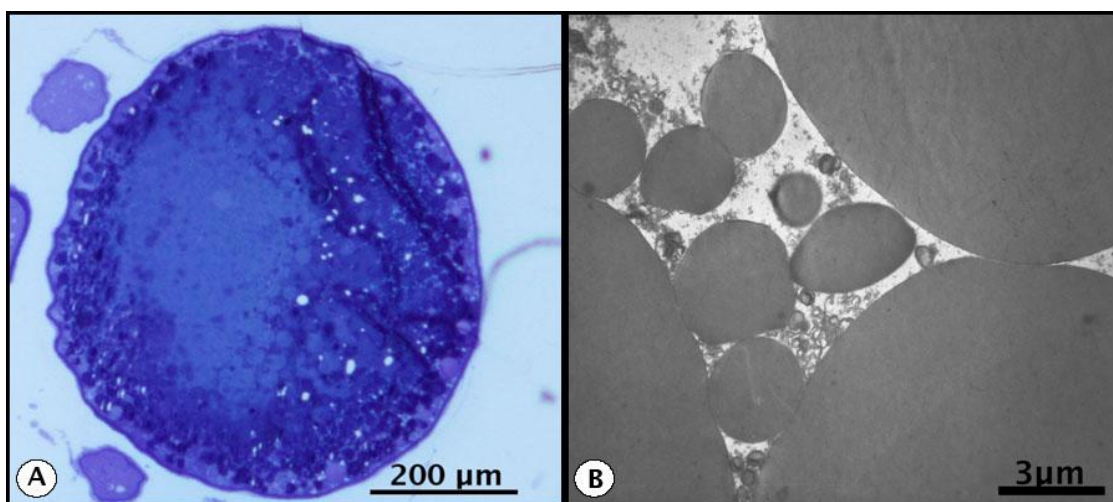


Fig. 5 Stage IV. Yolk bodies coalescence. a) LM; b) TEM.

The different histology approaches enabled to distinguish the different stages of the oocyte development and Goldner and AZAN staining emphasized some particular aspects (Fig. 6). In Goldner staining, the CA stained blue, while the yolk bodies stained red. This staining method is ideal to distinguish rapidly oocytes that have undergone vitellogenesis and started to centripetally accumulate yolk bodies within the cell.

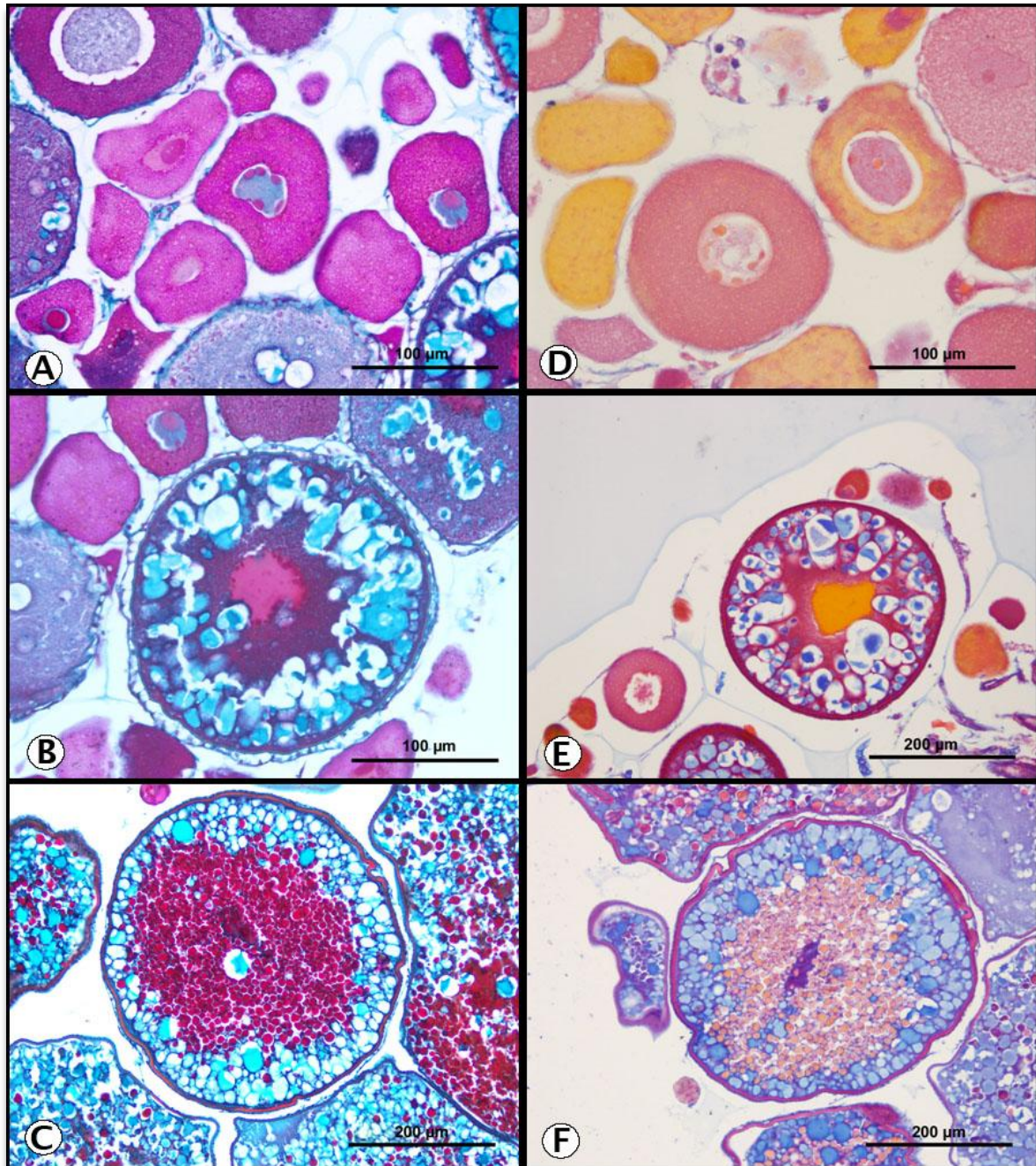


Fig. 6 Different histological staining. Goldner staining: A) Stage I; B) Cortical stage; C) Vitellogenic stage. AZAN staining: D) Stage I; E) Cortical stage; F) Vitellogenic stage.

AZAN staining was not described yet in oocytes, but revealed interesting aspects. In stage II, the GV stained orange as well as yolk bodies in stage III probably because they are basophilic. The CA were stained blue, probably because of their glycoprotein content.

IMUNOHISTOCHEMISTRY ASSAYS

The IHC had the aim to characterize the adhesion connections between the follicular cells and the oocyte. However some obstacles occurred as outlined below.

First the protocol was optimized in an epithelial zebrafish tissue, in order to test the specificity of the antibody to the species. After optimization we moved to then tissue of interest the oocyte starting from non-cultured oocytes and the antibody marked well the E-cadherine present in the adherens junctions in both tissues (Fig. 7).

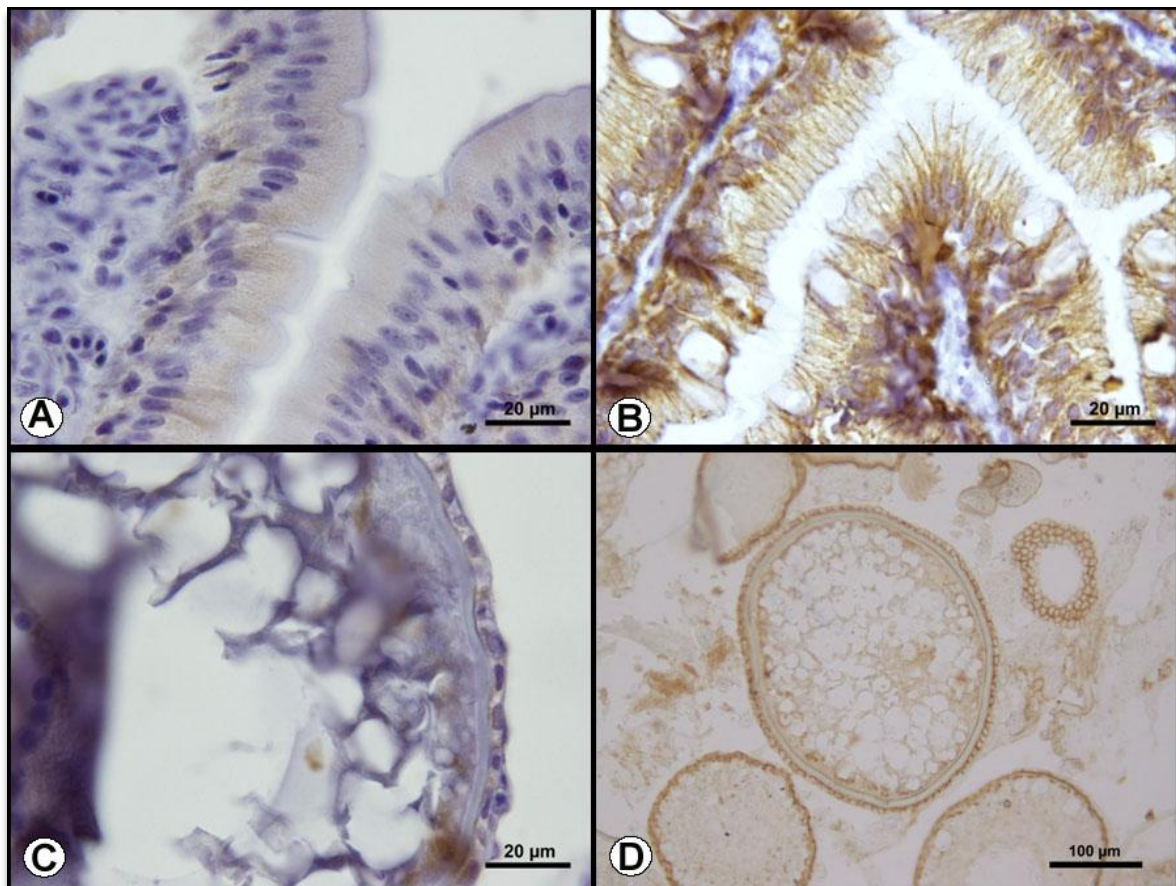


Fig. 7 Controls of IHC. A) Negative and B) positive control of the intestine of zebrafish. C) Negative and D) positive control of an oocyte of a zebrafish gonad without *in vitro* culture. Image of D is without the counterstaining with hematoxylin which obscures the antibody mark.

However, when the same procedure was applied with the fragments of zebrafish ovaries derived from 2 days *in vitro* culture, the antibody did not have a clear and precise binding as in the positive controls and it was not possible to distinguish the follicular cells membranes (Fig. 8).

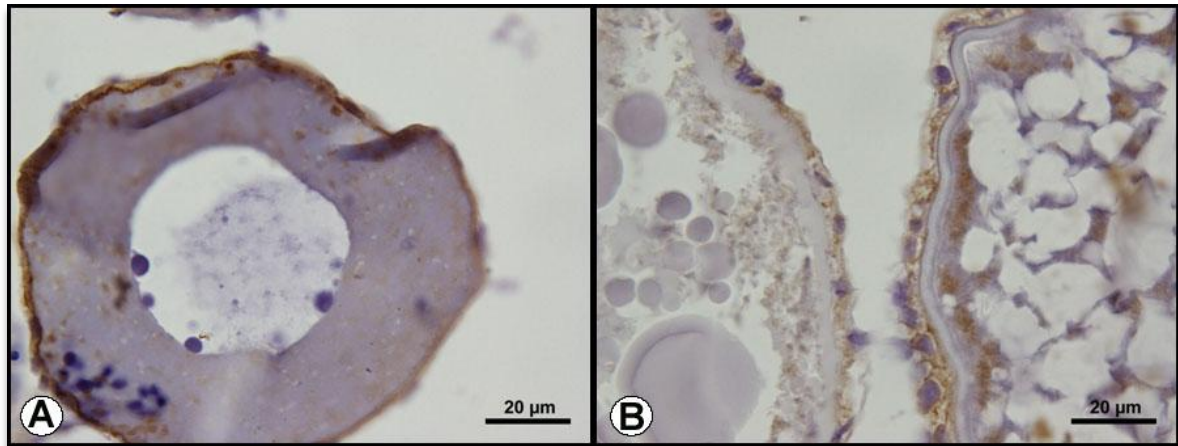


Fig. 8 IHC of oocytes after 2 days of culture. A) Stage I; B) Stage III/IV (left) and stage II (right).

CELL CULTURE CONDITIONS

Several techniques were tested for isolation of oocytes. The separation using needles is very slow and it caused high rates of damage. The use of pipettes for smooth aspiration is not aggressive and the follicular layers remain intact. Often enzymes as hyaluronidase, collagenase or trypsin (Guan et al., 2008b) are used to better separate the oocytes from the epithelial tissue. However this practice resulted in high degradation and low integrity of follicular cells. Therefore, an EDTA solution was applied after a first separation with pipetting and needles, followed by a second round of needles and aspiration. This procedure weakened the connections of the oocytes to the epithelial tissue, which helped the separation and did not damage the follicular cells (Fig. 9).

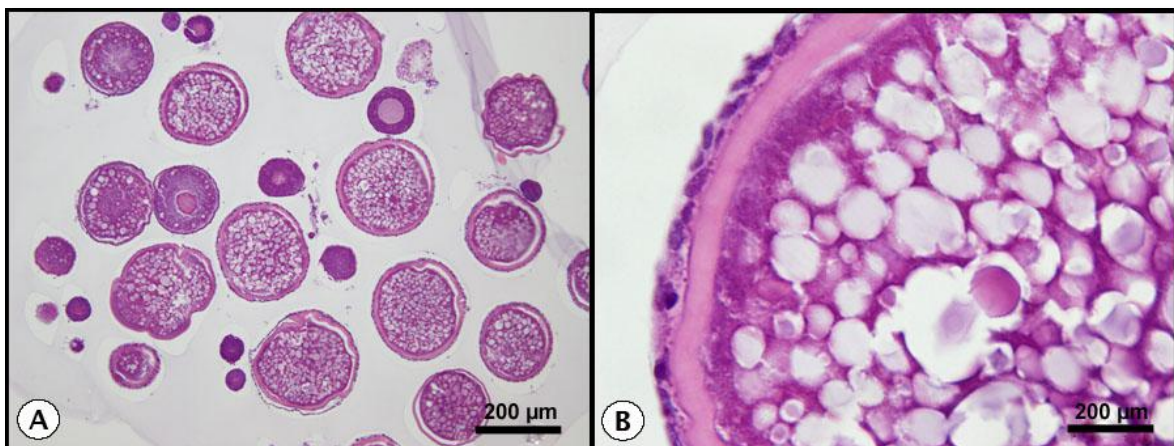


Fig. 9 Histology images (H&E) of the separation of the oocytes using aspiration, needles and EDTA 10mM.

Furthermore, some degenerative aspects were observed in the oocytes from the 75% concentration group (Fig. 10).

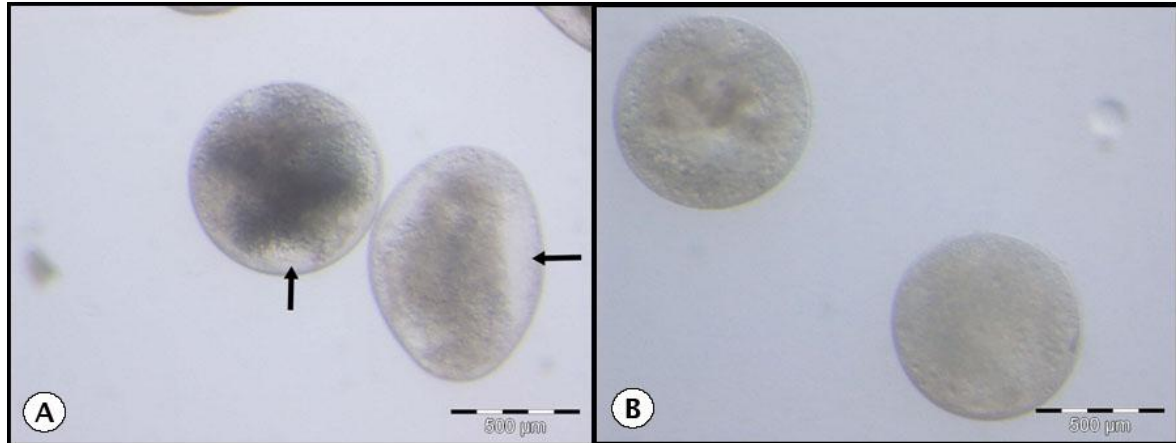


Fig. 10 Vitellogenic oocytes cultured at different medium concentrations. A) Leibovitz L-15 75%; signs of degeneration (arrows); B) Leibovitz L-17 90%.

Leibovitz L-15 is usually used in concentrations from 50% to 90% for zebrafish oocyte culture (Selman et al., 1993; Pang and Ge, 2002b; Guan et al., 2008a; Guan et al., 2008b; Seki et al., 2008). In our *in vitro* cultures, we tested a concentration of 75% and 90% (Seki et al., 2008) to verify the most appropriate to the culture. A significant increase in size was observed in the 75% concentration for vitellogenic oocytes at stage III ($p < 0.05$) compared to 90% medium (Fig. 11).

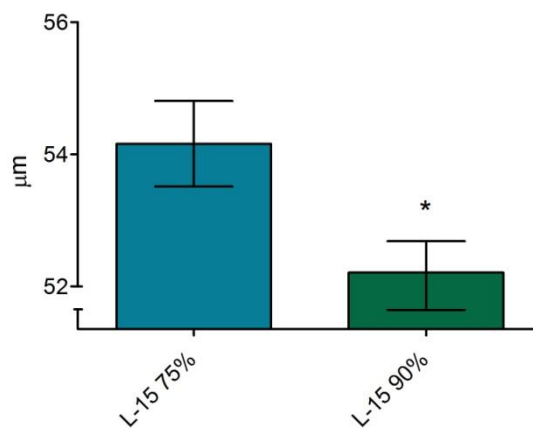


Fig. 11 Diameter of vitellogenic oocytes after culturing in different concentrations of Leibovitz L-15 medium. Oocytes grown in 75% medium increased significantly ($p < 0.05$) their diameter compared to those at 90% medium.

Every culture was routinely observed for contamination and discarded if detected. In some cases, we performed cytospin analyzes to characterize the type of contamination and to identify the sources (Fig. 12).

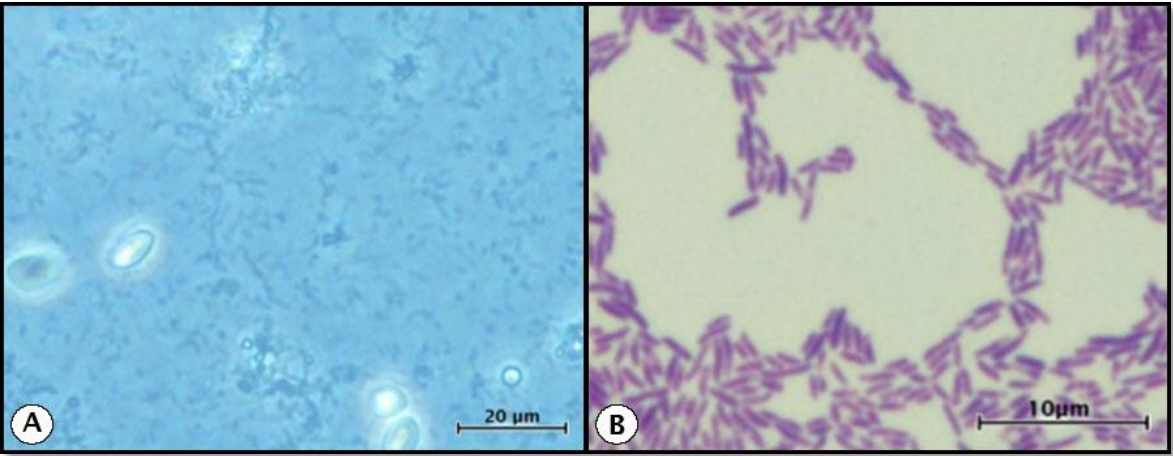


Fig. 12 Contamination detection: A) Culture plate under the inverted light microscope; B) Cytospin of bacterial contamination with Romanovsky staining.

VIABILITY AND MORPHOLOGICAL ALTERATION DURING OOCYTE CULTURE

The viability of the culture was verified with different methods: trypan blue staining (Fig. 14) and histological evaluation (Fig. 15). Trypan blue staining indicated viability between 50 and 80% for stage I, II, and III, which did not vary much over the course of six days of culture. The histological analysis revealed a similar degree of viability for all three stages. However, at day 6, especially oocytes of stage II and III/IV showed a lower viability in the histology representation. Though, the n of oocytes counted for stages III/IV was low (Fig. 13).

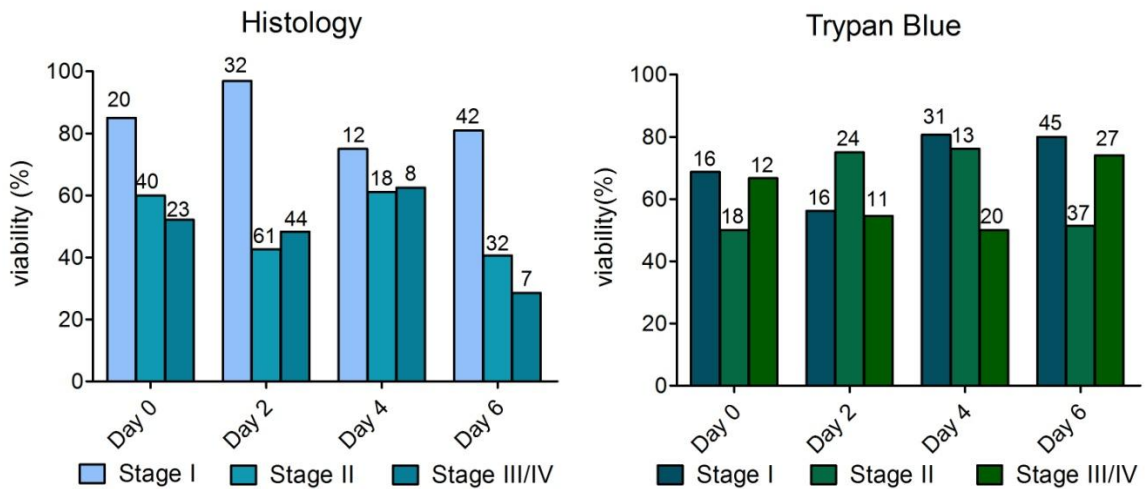


Fig. 13 Graphic representing the viability observed in the time course of isolated oocytes by paraffin histology and trypan blue staining test. Numbers above columns represents the number of oocytes counted.

These results may reflect that oocytes degenerated over the time of culture and that the manipulation of those for histology procedures collapsed fragile cells, which consequently did not appear in images.

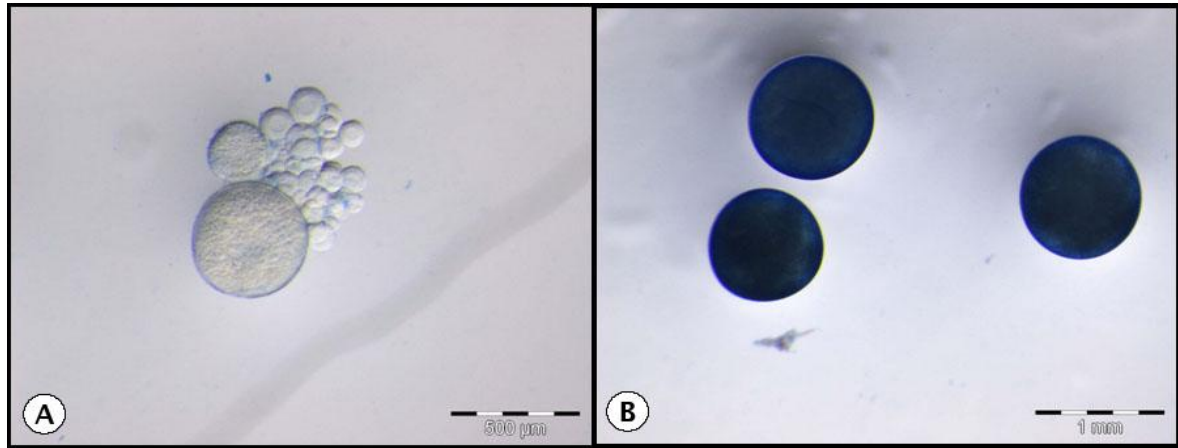


Fig. 14 Trypan blue viability test. A) At stages I, II and III after trypan blue treatment; B) Unviable oocytes of stage III and IV after trypan blue staining.



Fig. 15 Histological evaluation of oocyte viability (H&E). A) Viable oocytes; B) Unviable oocytes.

In the following, results are presented from a time course experiment (Fig. 16). The morphological characteristics of the oocytes of stage I and II did not change over the course of six days as analysed by standard histological staining. Oocytes of stage III seemed to be more instable to *in vitro* culture in the establish conditions (with oocyte isolation and separation). At day 6, a possible degradation was observed as an excessively increase of vitelline envelope, a detachment of the follicular layer and liquefied yolk globules. Furthermore, the zp of the oocytes seemed very increased and not very preserved.

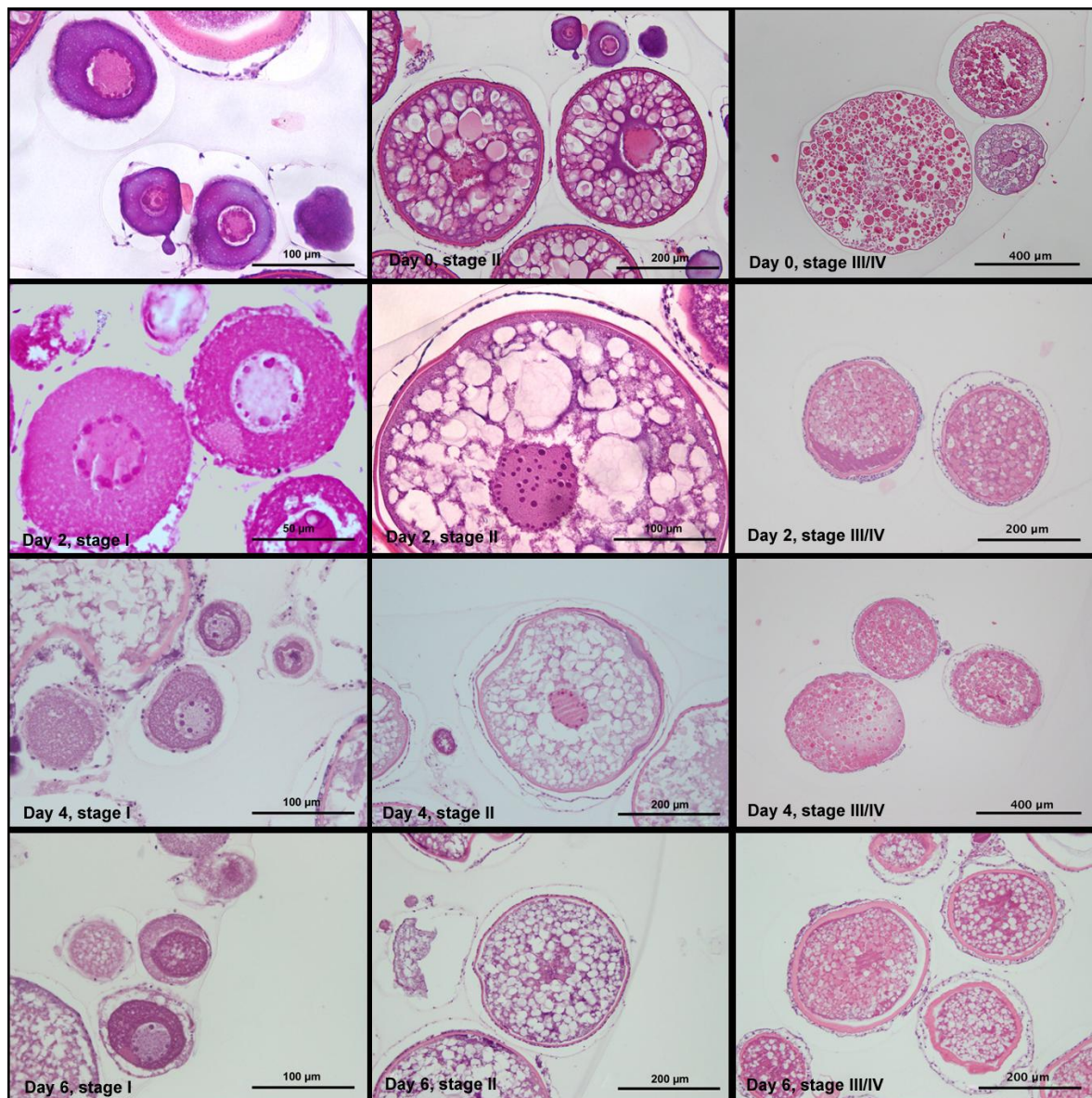


Fig. 16 Histology results of the time course with isolated oocytes at stage I, II and III/IV (H&E).

For characterizing further details of the potential morphological alterations of oocytes in *in vitro* cultures, another time course was performed for the use with semi-thin slices fixed in Epoxy resins. Here, the presence of large yolk bodies hampered the post fixation of the samples and it was tested the different times of post fixation. The overnight showed better results than using just 2h of post fixation (Fig. 17).

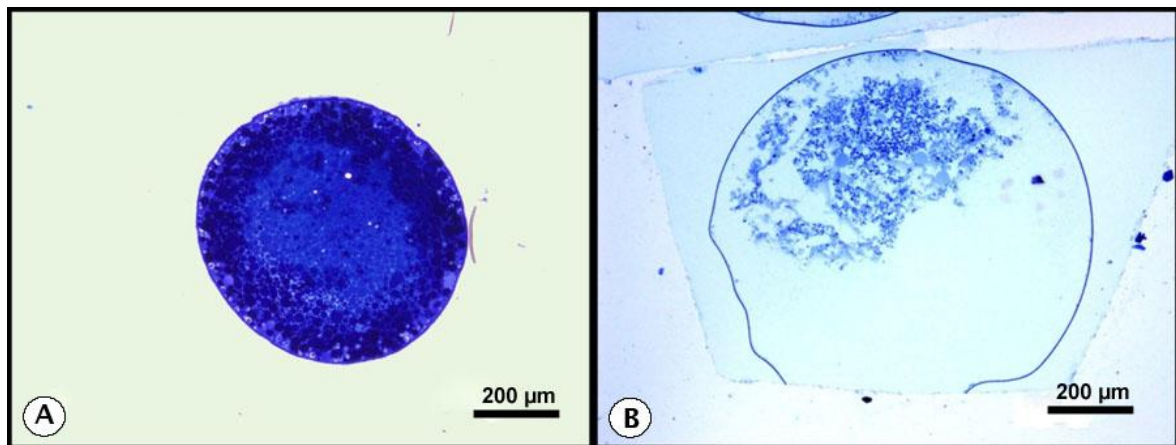


Fig. 17 LM of an isolated vitellogenic oocyte with overnight of post fixation. (A) and 2h of post fixation (B).

The oocytes of the stages I and II were well preserved during the culture period of 4 days, while some signs of degeneration were visible for stage II at day 4, and for stage III at day 2 and 4 (Fig. 18). However, these alterations could also originated from the technical processing of the histological slides. It was striking that follicular cells were frequently disconnected or even absent.

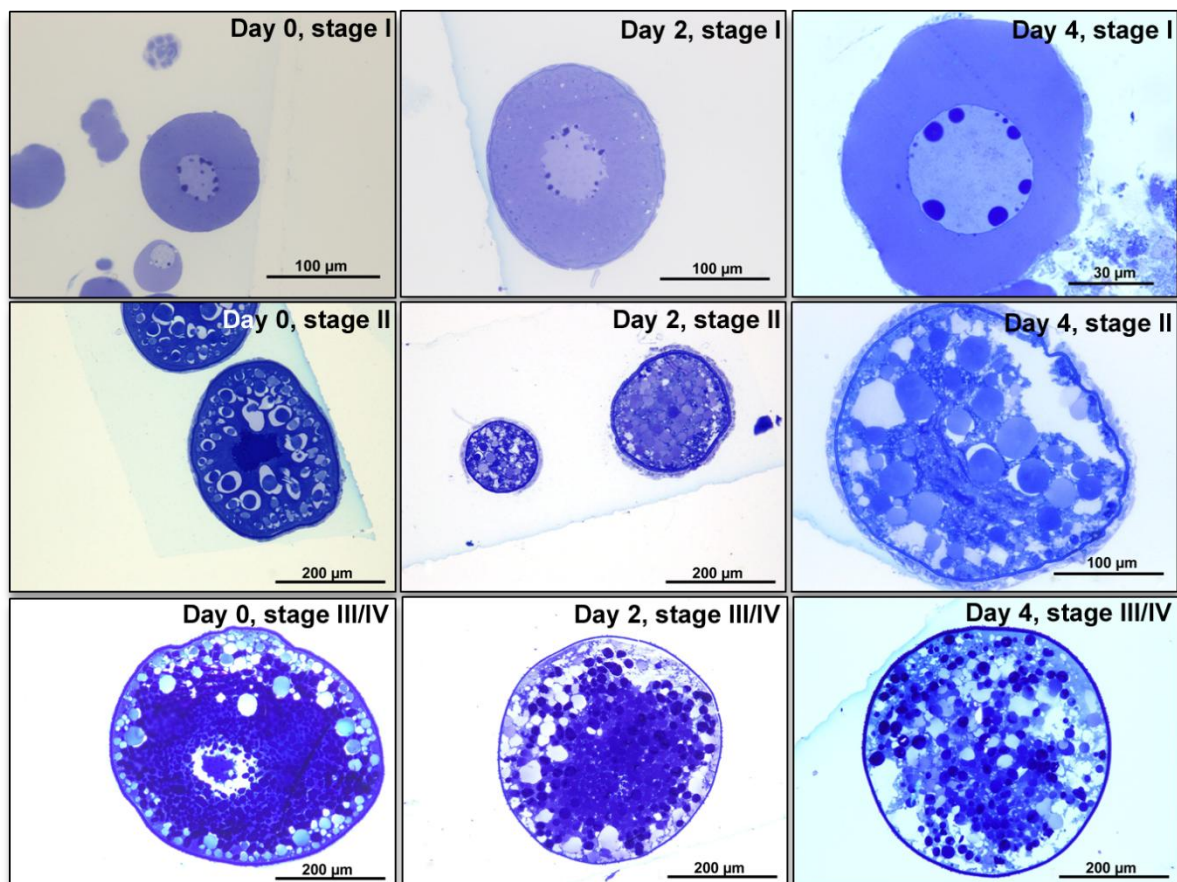


Fig. 18 LM of isolated oocytes of zebrafish cultured for 4 days (time course) with overnight post fixation.

For that reason, in the next experiment fragments of ovaries were used in the *in vitro* culture. Herewith, a better integrity of follicular cells was observed in the ultrastructural analyzes (Fig. 19). In four days of culture, oocytes had a similar viability to those that were cultured separately, but the follicular layers were more preserved.

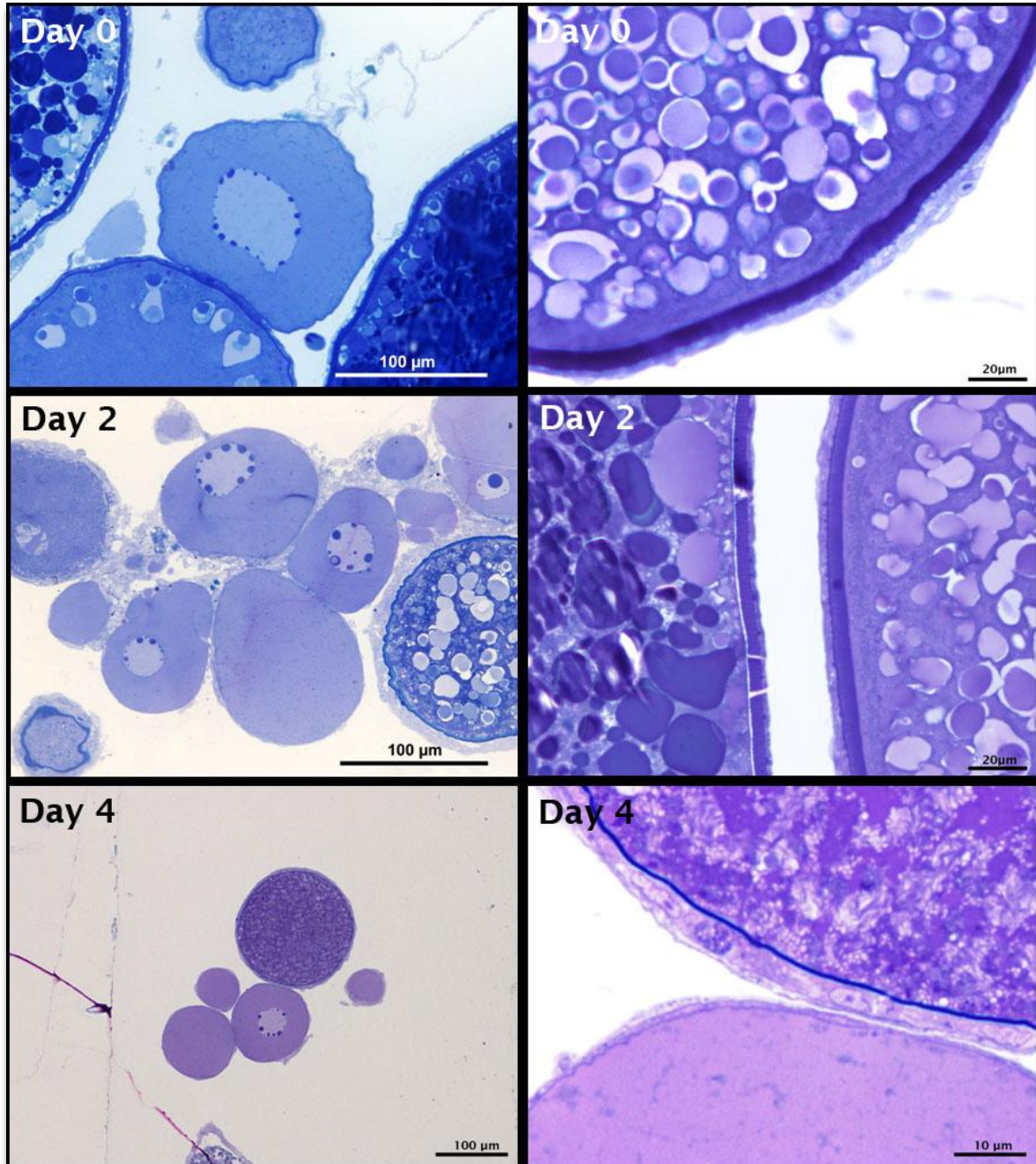


Fig. 19 LM of the fragments of zebrafish ovaries cultured for 4 days (time course). Right images show the follicular layers with more details.

The presence of possible lipid droplets was observed under the light microscope at a high resolution (x100) in follicular layers of oocytes of day 2, but not at day 0. At day 4 they were frequently destroyed (Fig. 20).

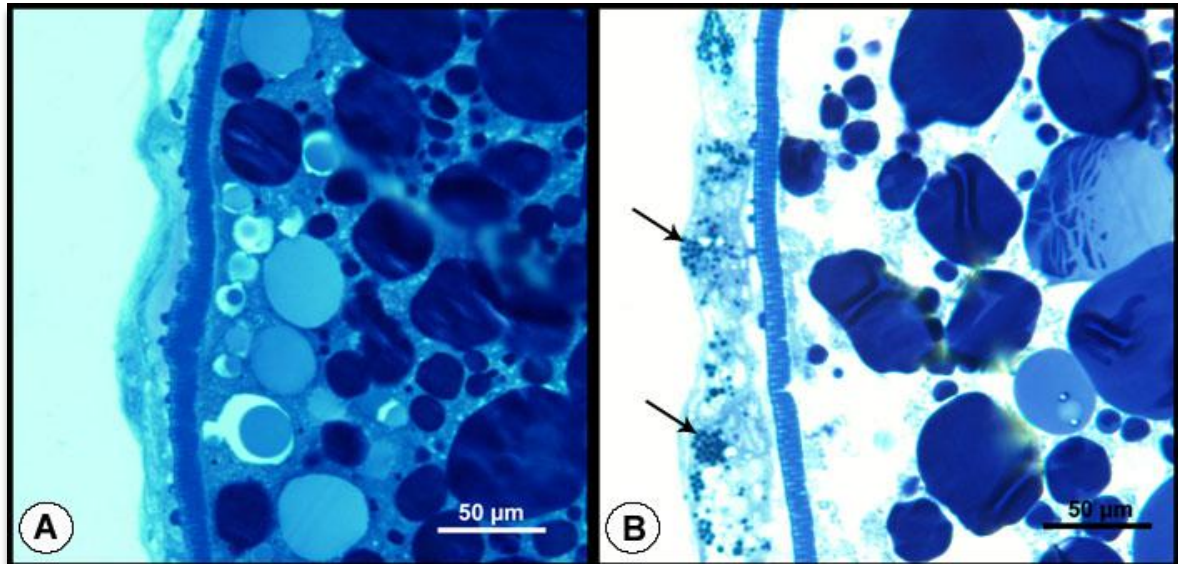


Fig. 20 Presence of lipid droplets in follicular cells (LM). A) Vitellogenic oocyte at day 0 where the follicle cells do not show presence of lipid droplets. B) A vitellogenic oocyte after 2 days of culture, where the follicle cells exhibit the presence of lipid droplets (arrows).

Likewise the observations in isolated oocytes, all oocyte stages in fragments at day 4 revealed good aspects. As last analysis of morphological alterations in the *in vitro* culture period, TEM was performed, where it was possible to investigate the follicular cells and oocyte conditions, structures and evolution over the time with more detail.

On Day 0, all oocytes stages analyzed (I, II and III) were viable and very intact and the principal ultrastructures that characterize these cells were present. In stage I the zp begins to form. Firstly the microvilli develop and then dense material deposits between those structures. On Day 2, the stage I reveals activity with the continuously development of the vitelline envelope, *Balbiany bodies* and *nuages*. Finally, at day 4, stage I remains with high viability still showing intracellular activity (Fig. 21).

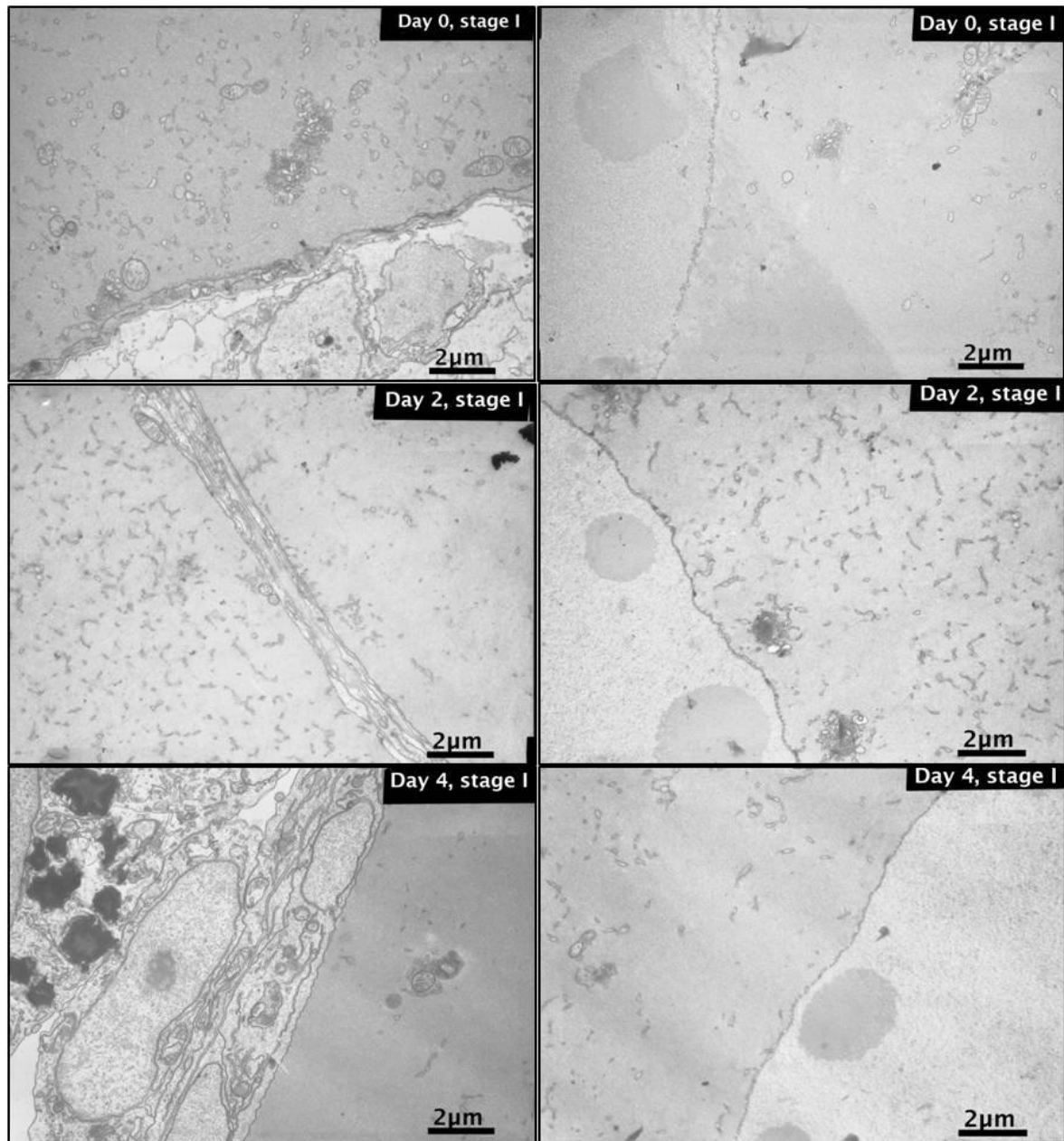


Fig. 21 TEM of oocytes at stage I of the time course. Left images represent the periphery of the oocytes along the time and right images the inside of the oocytes.

Oocytes at cortical stage were very preserved and easily characterized at day 0. After 2 days of culture, good viability was observed with well formed membranes and with follicular cells development. On Day 4, stage II was not so preserved as before. It was possible to see some disintegration of the ER and the appearance of large vesicles that may be vacuoles. Thecal cells are sometimes detached and destroyed (Fig. 22).

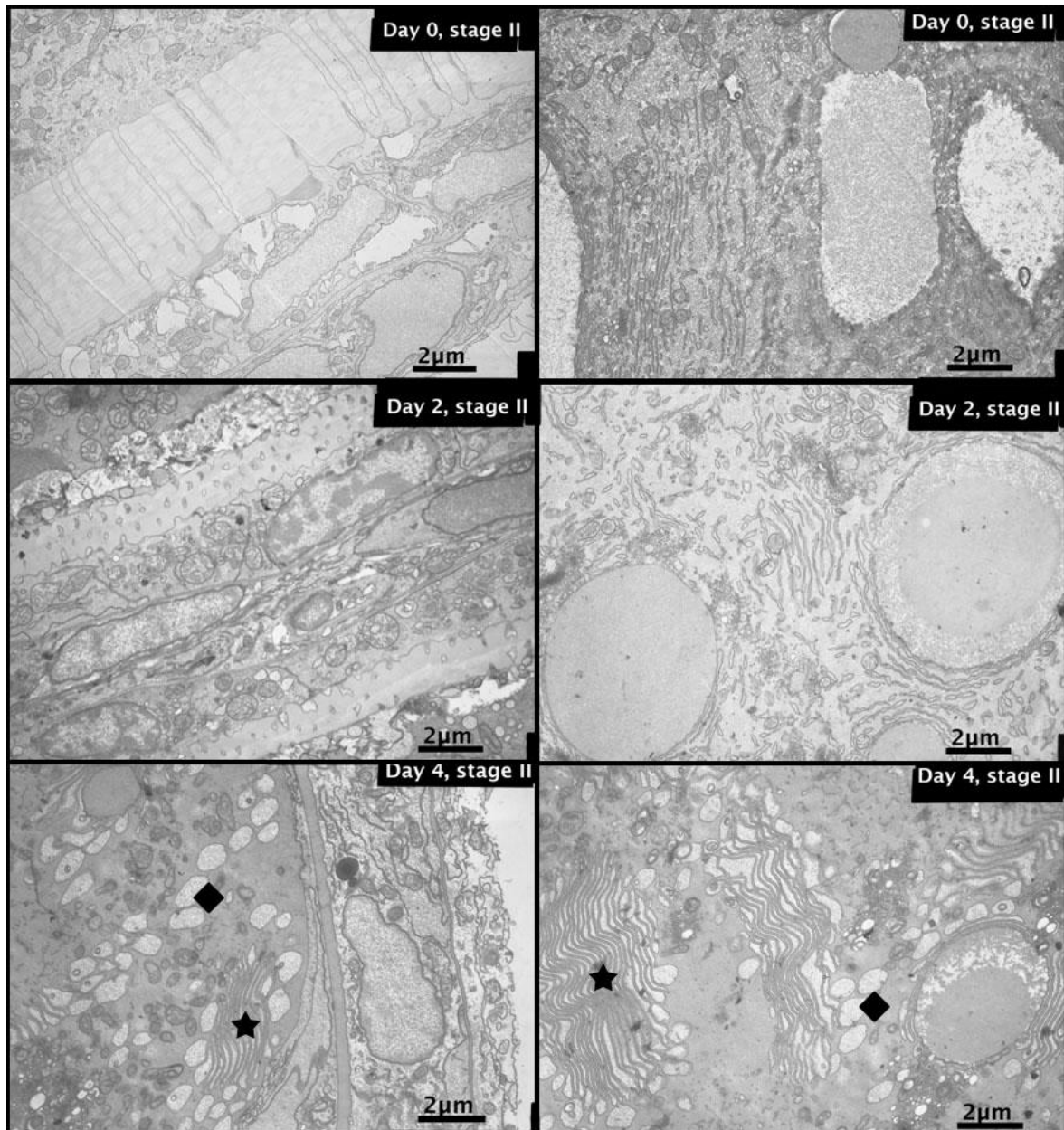


Fig. 22 TEM of stage II oocytes of the time course. Left images represent the periphery of the oocytes, right images the inside of the oocytes. ♦ Vacuoles; ★ ER disintegration.

At day 0 and day 2 of the culture, vitellogenic oocytes were intact. However, the visualization for TEM is commonly hampered because of the enrichment of this stage in lipids either within the oocyte or follicular cells. At day 2, as it was already seen in the semi-thin sections in LM, the presence of lipid droplets in follicular cells was noted, which could due to technical reasons result in a worse visualization. At day 4, the oocytes of stage III were frequently wrecked without mitochondrias inside, with the two follicular layers destroyed and detached from the zp. The microvilli in the zp had material sequestered (Fig. 23).

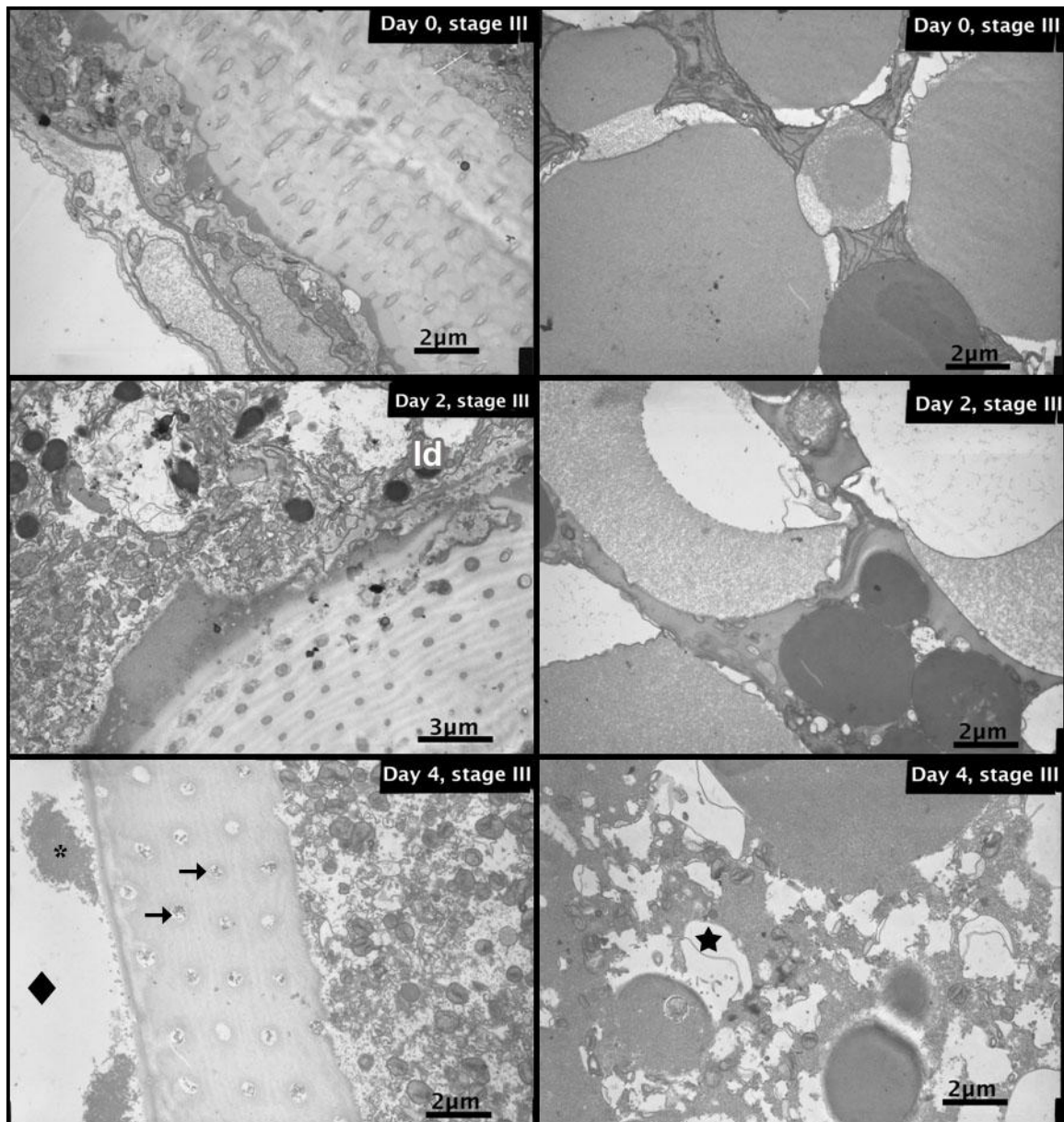


Fig. 23 TEM of vitellogenic stages along 4 days of culture. Left images represent periphery of the oocytes, right images the inside of the oocytes. ld) lipid droplets; * unkown electrodense material; ♦ detachment of follicular layers; arrows indicate sequestered material in the microvilli; ★vacuolization.

EXPOSURE TO REPRODUCTIVE AND STRESS HORMONES

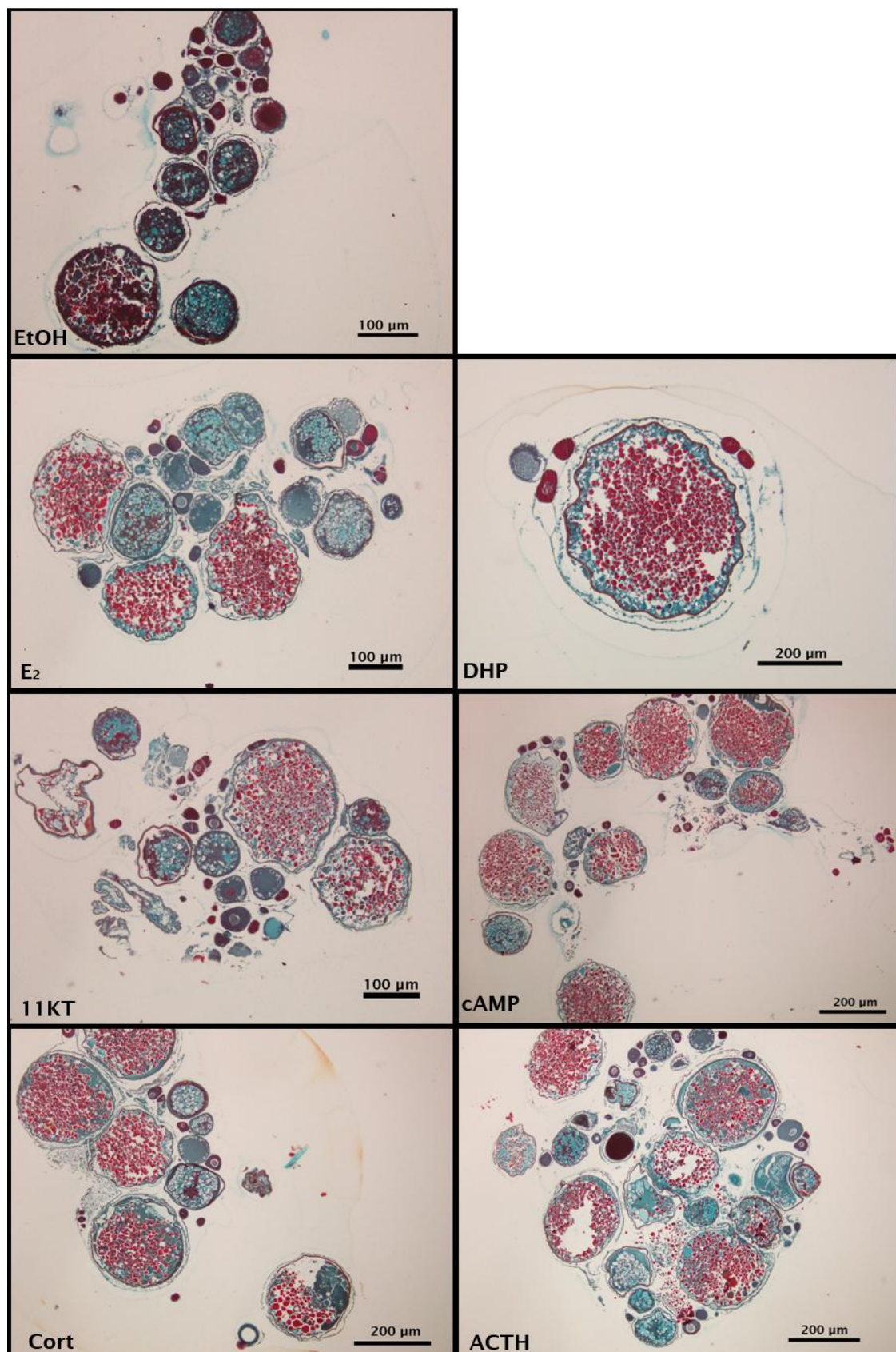


Fig. 24 Goldner staining of oocytes - exposed for 2 days in fragments to different hormones (LM).

Goldner staining did not show any differences of the exposure to the different hormones compared to the control group at the normal histological level (Fig. 24).

Electron microscopy

A qualitative analysis of TEM results was performed in order to achieve the main alterations of the oocytes exposed to the different substances. Several aspects were characterized. Six individuals were qualitatively analyzed to learn about the consistency of the occurrence of potential effects.

For Stage I the following parameters were evaluated: the presence/ abundance of mitochondria inside the oocyte, the reticulation of GV, the presence/abundance Golgi apparatus, the presence/proliferation of rER and sER, the development of zp, the development of a first follicular layer, the “pre-granulosa layer” (below the basal lamina), and a second follicular layer (above the lamina layer), called here pre-thecal layer (Table 1).

In stage I, DHP seemed to induce the formation of the pre-granulosa layer (70% present) as revealed by the increased presence of pre-granulosa layer compared to the control. cAMP had an apparent decrease in the pre-thecal cell layer development (66.7% absent) (Fig. 25). Oocytes of stage I exposed to Cort revealed a high proliferation of sER (33.3% abundant + 66.7% very abundant) that was not observed in control group. Cort also induced a higher GV reticulation (80% present + 20% abundant). ACTH did not demonstrate alterations compared to the control, however the oocytes were frequently less preserved (viability was 66.7%) (Fig. 26).

For Stage II, the following endpoints were characterized: the presence of mitochondria dispersed or in agglomerates, the deformation of CA, the presence/abundance of ER around the CA, the presence of rER in two different forms (stack and anullate lamellae), the presence/abundance of sER, the presence/ abundance of Golgi apparatus, the development of the zp, the development of granulosa cells layer, the development of thecal cells layer and the presence/abundance of unknown electron dense material near above the zp (Table 2).

Oocytes of stage II exposed to E₂ had a higher proliferation of the ER around the CA (20% present + 80% abundant) and also seemed to have slightly increase of granulosa cells development (20% present, 60% abundant, 20% very abundant) (Fig. 27) comparing to the control. The oocytes exposed to cAMP revealed a higher proliferation of sER distributed all over the ooplasm (100% abundant). Once again, the stress hormones

showed more alterations. Cort stimulated a very high proliferation of rER in a form similar to anullate lamellae (100% very abundant) and zp seems more developed (33.3% present + 33.3% abundant + 33.3% very abundant). ACTH induced cell death in almost all oocytes observed in TEM and showed no viable organelles inside the oocyte and in follicular layers (viability was 16.7%) (Fig. 28).

Table 1 Contingency analysis of morphological alterations observed under TEM in oocytes of stage I and respective follicular cells exposed to the chosen hormones.

STAGE I		Ctrl(%)	E ₂ (%)	11KT (%)	DHP (%)	cAMP (%)	Cort (%)	ACTH (%)
mitochondria	absent	16.7	25.0	0.0	40.0	33.3	16.7	40.0
	present	83.3	75.0	100.0	40.0	66.7	50.0	60.0
	abundant	0.0	0.0	0.0	20.0	0.0	33.3	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GV reticulation	absent	40.0	50.0	50.0	80.0	83.3	0.0	75.0
	present	60.0	50.0	50.0	20.0	0.0	80.0	0.0
	abundant	0.0	0.0	0.0	0.0	16.7	20.0	25.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Golgi apparatus	absent	66.7	100.0	83.3	40.0	50.0	33.3	60.0
	present	33.3	0.0	16.7	60.0	50.0	66.7	0.0
	abundant	0.0	0.0	0.0	0.0	0.0	0.0	20.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
sER	absent	16.7	0.0	0.0	20.0	16.7	0.0	20.0
	present	50.0	75.0	83.3	80.0	83.3	0.0	60.0
	abundant	33.3	0.0	16.7	0.0	0.0	33.3	20.0
	very abundant	0.0	25.0	0.0	0.0	0.0	66.7	0.0
rER	absent	100.0	100.0	100.0	100.0	100.0	83.3	100.0
	present	0.0	0.0	0.0	0.0	0.0	16.7	0.0
	abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
zp development	absent	83.3	25.0	100.0	100.0	100.0	50.0	75.0
	present	16.7	75.0	0.0	0.0	0.0	50.0	50.0
	abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pre-granulosa layer	absent	91.7	87.5	90.0	30.0	91.7	91.7	80.0
	present	8.3	12.5	10.0	70.0	8.3	8.3	20.0
	abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pre-thecal layer	absent	0.0	25.0	0.0	0.0	66.7	16.7	20.0
	present	100.0	75.0	100.0	100.0	33.3	83.3	80.0
	abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
perinucleous	absent	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	present	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
viability		100.0	100.0	100.0	100.0	100.0	91.7	66.7

Table 2 Contingency analysis of morphological alterations observed under TEM in oocytes of stage II and respective follicular cells exposed to the chosen hormones.

STAGE II		Ctrl (%)	E ₂ (%)	11KT (%)	DHP (%)	cAMP (%)	Cort (%)	ACTH (%)
mitochondria agglomerate	absent	33.3	80.0	66.7	40.0	100.0	50.0	16.7
	present	50.0	0.0	0.0	20.0	0.0	16.7	0.0
	abundant	16.7	0.0	16.7	40.0	0.0	33.3	16.7
	very abundant	0.0	20.0	16.7	0.0	0.0	0.0	0.0
mitochondria (dispersed)	absent	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	present	33.3	100.0	33.3	80.0	100.0	100.0	16.7
	abundant	66.7	0.0	50.0	20.0	0.0	0.0	16.7
	very abundant	0.0	0.0	16.7	0.0	0.0	0.0	0.0
CA deformation	absent	100.0	60.0	83.3	40.0	80.0	100.0	50.0
	present	0.0	0.0	16.7	20.0	20.0	0.0	0.0
	abundant	0.0	40.0	0.0	40.0	0.0	0.0	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ER around Cortical Alveoli	absent	50.0	0.0	83.3	40.0	70.0	0.0	33.3
	present	50.0	20.0	16.7	60.0	30.0	83.3	16.7
	abundant	0.0	80.0	0.0	0.0	0.0	16.7	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
rER	absent	66.7	100.0	50.0	60.0	80.0	0.0	33.3
	present	0.0	0.0	50.0	20.0	0.0	0.0	0.0
	abundant	33.3	0.0	0.0	20.0	20.0	0.0	16.7
	very abundant	0.0	0.0	0.0	0.0	0.0	100.0	0.0
sER	absent	0.0	16.7	16.7	60.0	0.0	100.0	0.0
	present	100.0	66.7	83.3	20.0	0.0	0.0	0.0
	abundant	0.0	16.7	0.0	20.0	100.0	0.0	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	33.3
Golgi apparatus	absent	16.7	50.0	16.7	20.0	20.0	0.0	0.0
	present	66.7	50.0	83.3	60.0	60.0	50.0	50.0
	abundant	16.7	0.0	16.7	20.0	20.0	50.0	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
zp development	absent	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	present	83.3	60.0	50.0	66.7	60.0	33.3	66.7
	abundant	16.7	40.0	50.0	33.3	40.0	33.3	16.7
	very abundant	0.0	0.0	0.0	0.0	0.0	33.3	16.7
granulosa cells	absent	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	present	50.0	20.0	50.0	50.0	40.0	50.0	33.3
	abundant	50.0	60.0	50.0	50.0	60.0	50.0	33.3
	very abundant	0.0	20.0	0.0	0.0	0.0	0.0	0.0
thecal cells	absent	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	present	16.7	0.0	16.7	0.0	100.0	33.3	0.0
	abundant	83.3	100.0	83.3	100.0	0.0	66.7	50.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
electrodense material (outside zp)	absent	100.0	100.0	100.0	66.7	83.3	80.0	33.3
	present	0.0	0.0	0.0	33.3	16.7	20.0	0.0
	abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	very abundant	0.0	0.0	0.0	0.0	0.0	0.0	0.0
viability		100.0	100.0	83.30	100.0	100.0	91.70	16.7

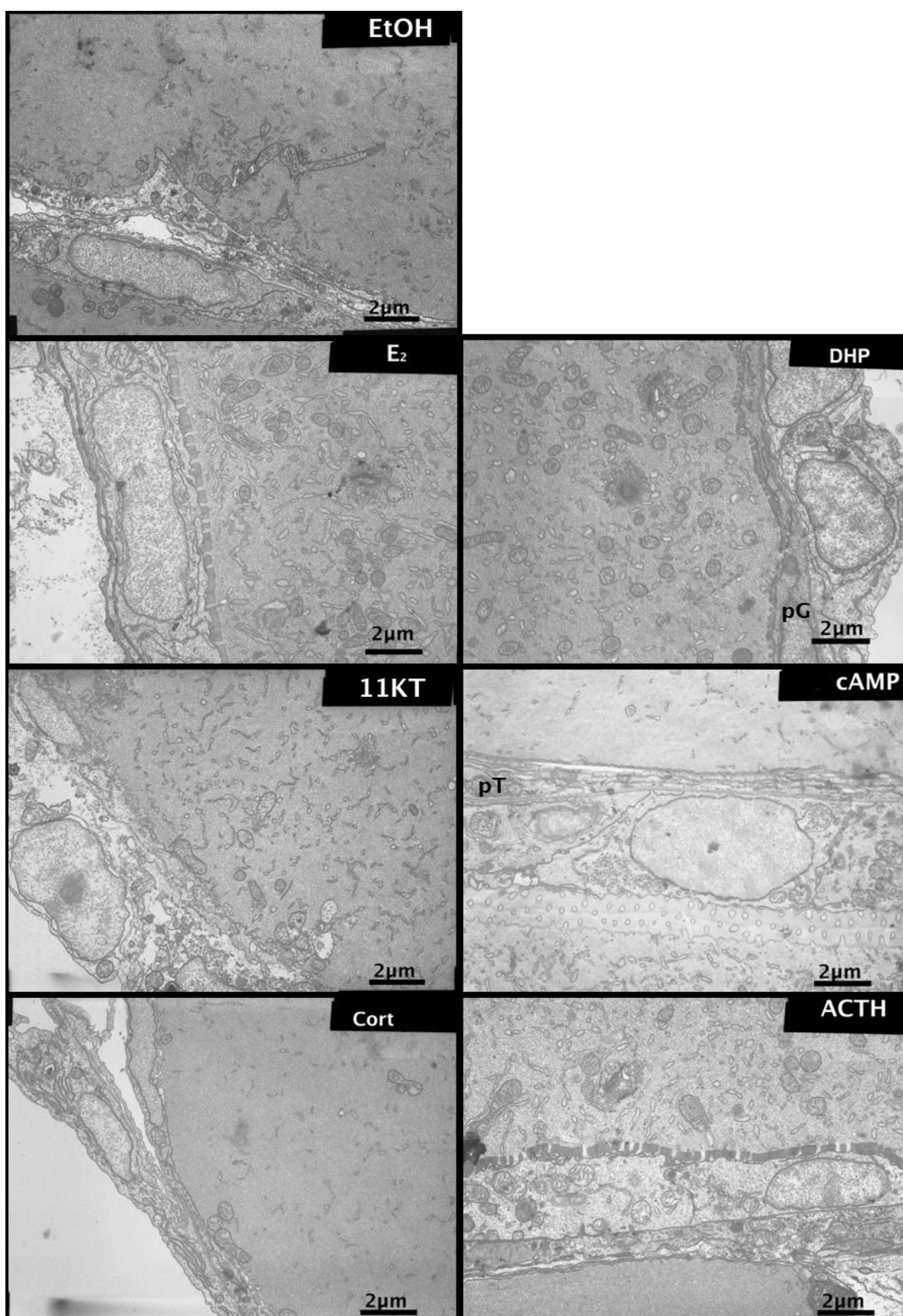


Fig. 25 Periphery of oocytes at stage I exposed for 2 days to the set of the chosen hormones (TEM). Pre-granulosa cell (pG); pre-thecal cell (pT).

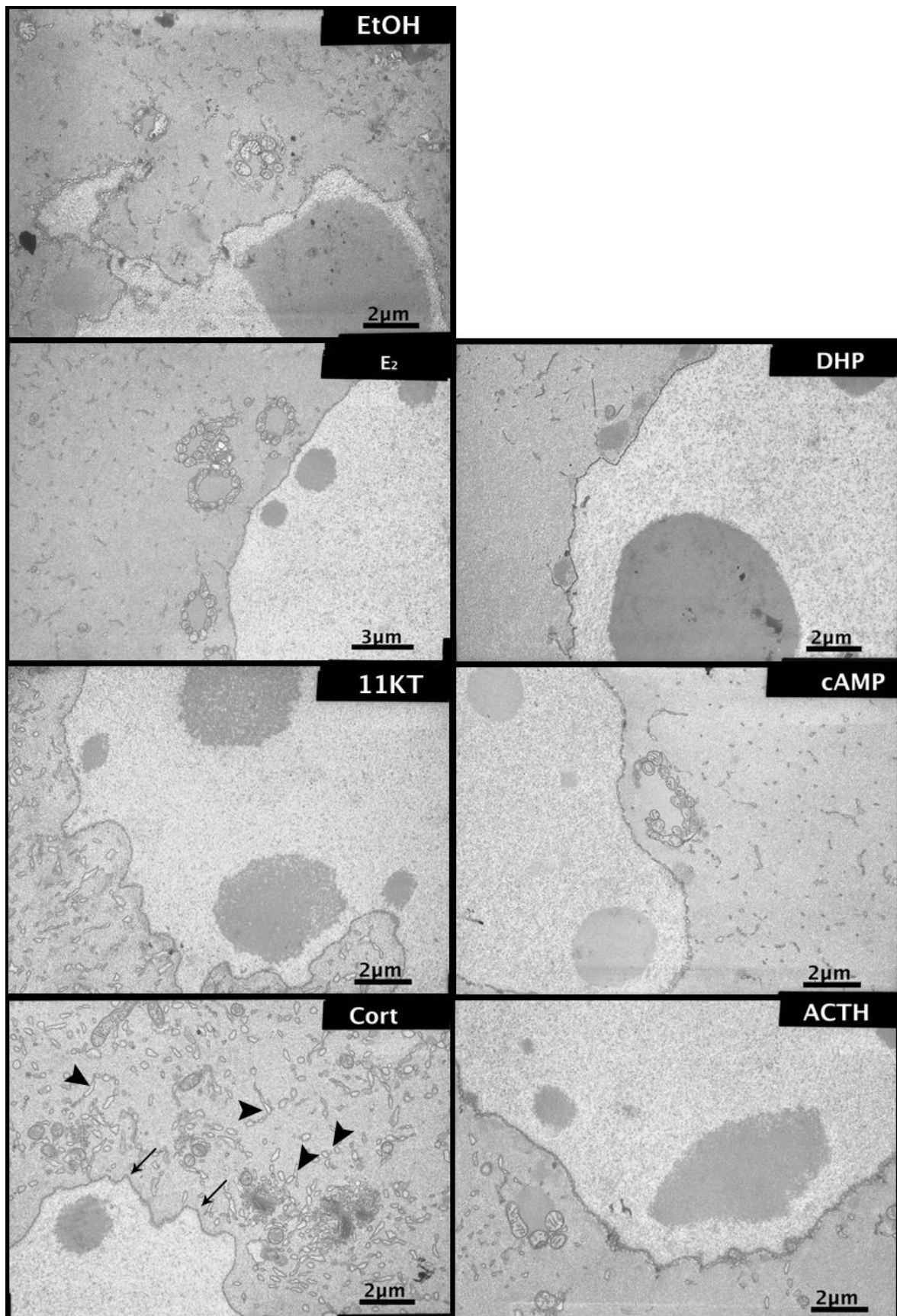


Fig. 26 Inside of oocytes at stage I exposed for 2 days to the set of the chosen hormones (TEM). Development of sER (arrowheads) and GV reticulation (arrows).

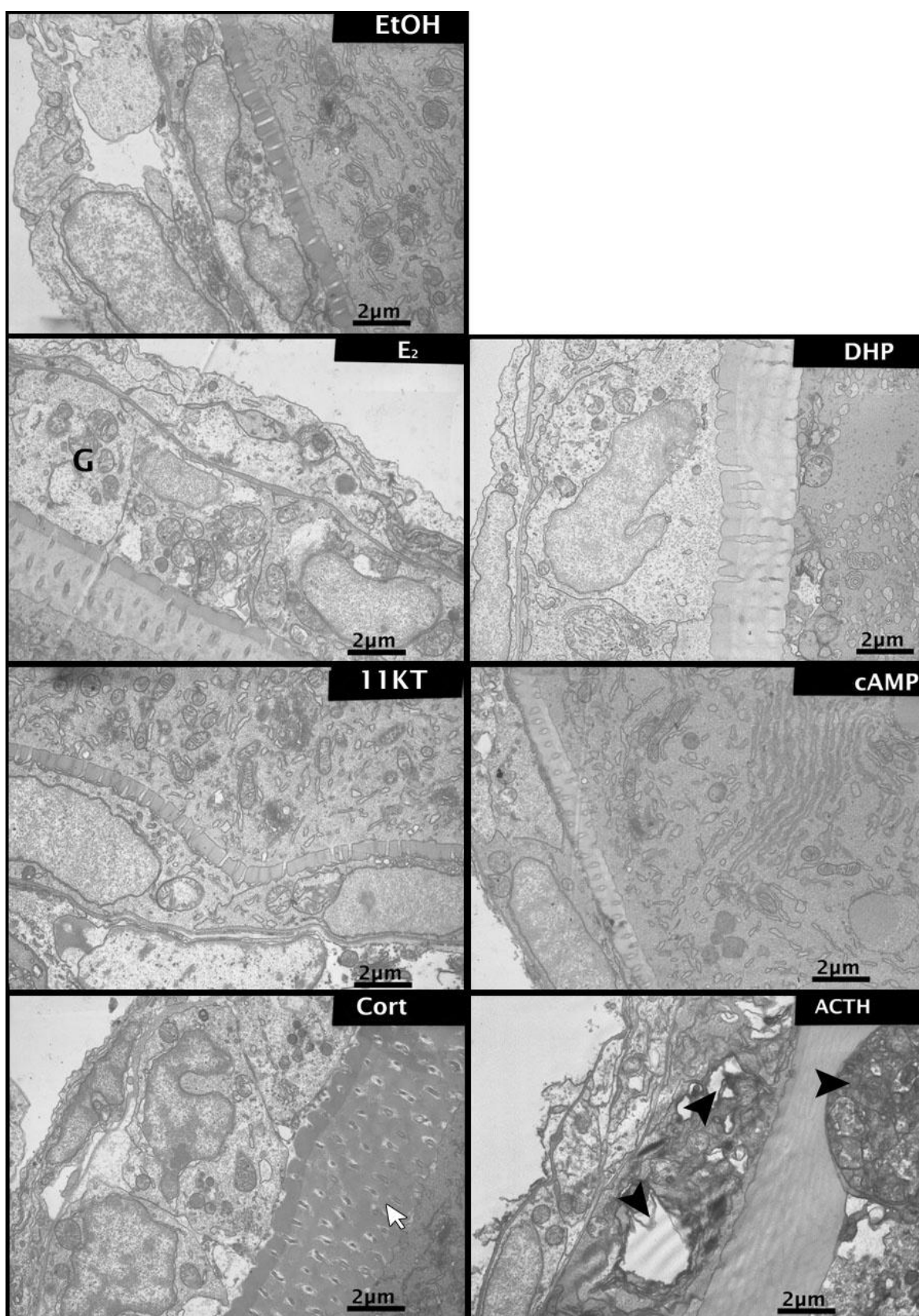


Fig. 27 Periphery of oocytes at stage II exposed for 2 days to the chosen hormones (TEM). Granulosa Cell layer (G). Cell degradation (arrowheads); zp development (white arrow).

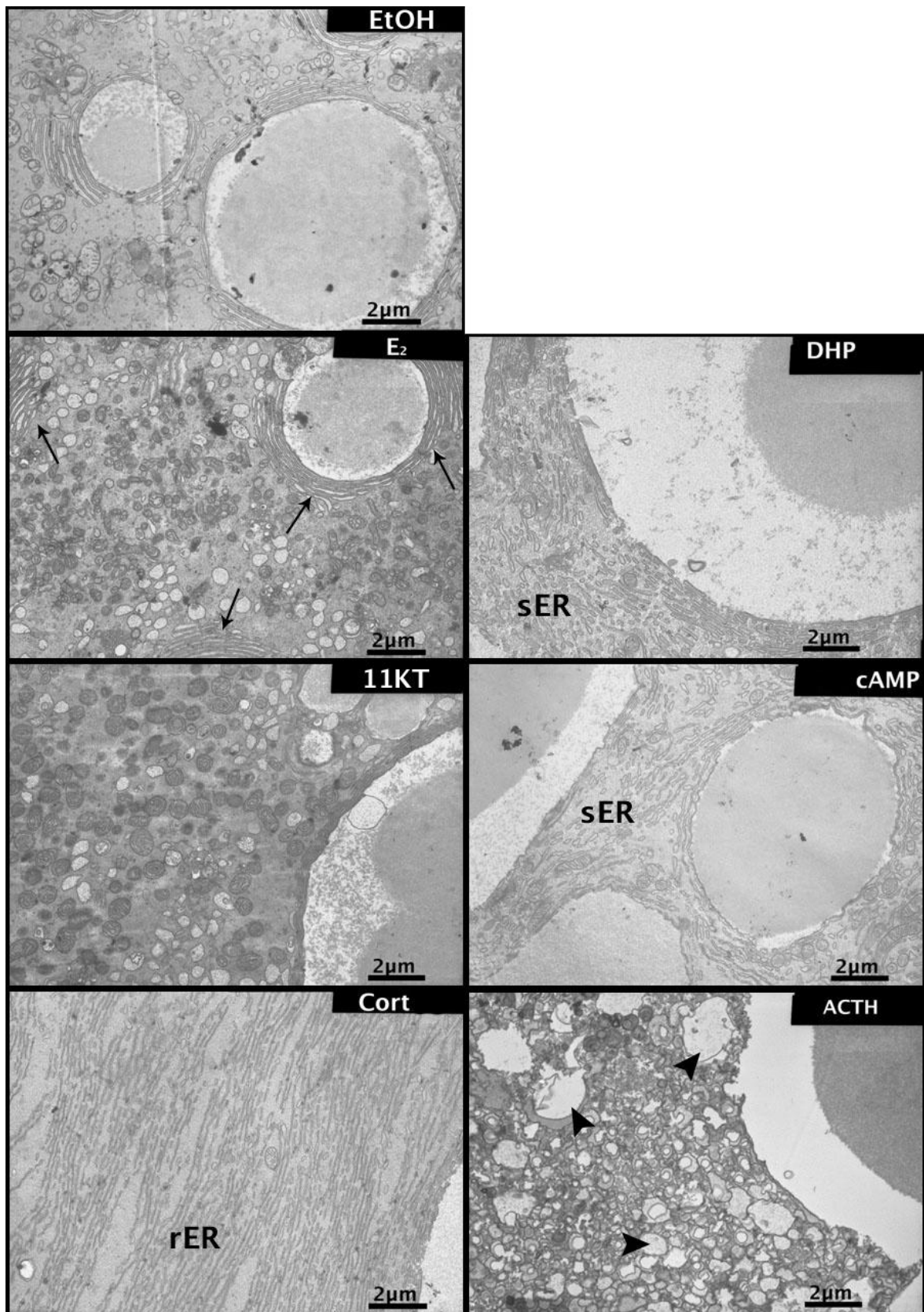


Fig. 28 Inside of oocytes at stage II exposed for 2 days to the chosen hormones (TEM). Development of ER around CA (arrows); development of sER in the cytoplasm (sER); vacuolization (arrowheads).

STEREOLOGY

Volume- weighted mean value (V_v)

Nuclear volumes of granulosa cells of the different exposed follicles were measured by PSI method (Fig. 29).

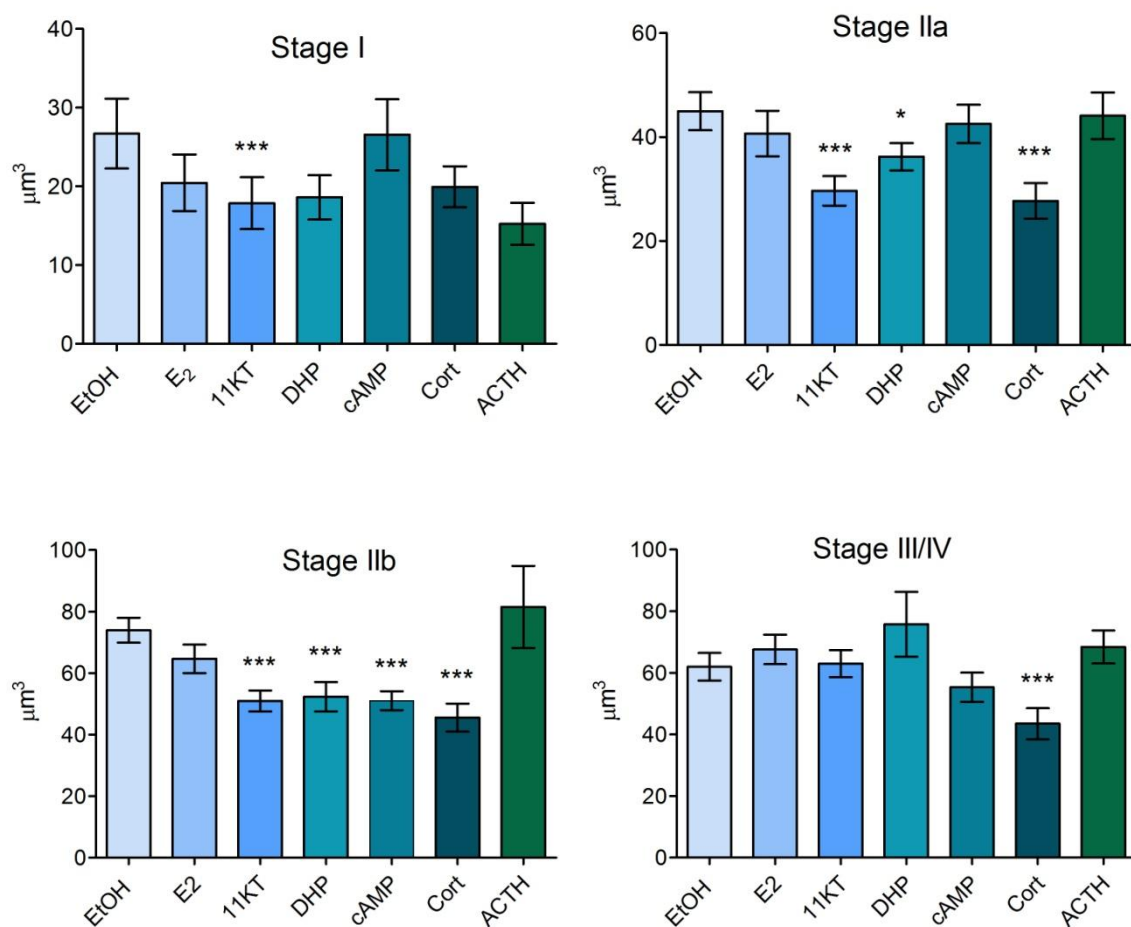


Fig. 29 Results of the nuclear weighted mean value of granulosa cells exposed to the different hormones. (*** - p < 0.001; * - p < 0.05).

For stage I, only 11KT altered the nuclear V_v of granulosa cells with a significant decrease of the nuclear volume compared to the control. Stage II was divided in two categories according to the abundance of CA. Significant alterations in the V_v of granulosa nuclei of the follicles exposed to 11KT, DHP and Cort were observed for the early CA stage (stage IIa). In the late CA stage (stage IIb), the nuclear volume of the granulosa cells were decreased in the follicles exposed to 11KT, DHP, cAMP and Cort. For

vitellogenic stages (III/IV), only the granulosa nuclear V_V decreased in the Cort exposure group.

While analysing the nuclear volumes of the control groups of the different oocyte-follicle stages, it was observed that the nuclear volume of granulosa cells increased from stage I to stage IIb and then decreased from IIb to III. After this, the nuclear volume is apparently maintained (Fig. 30).

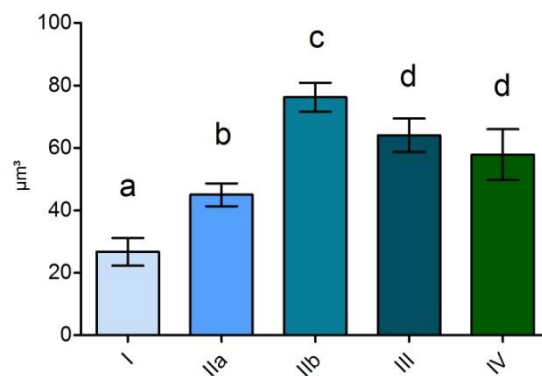


Fig. 30 Differences in the nuclear V_V of granulosa cells in control oocytes. Different letters represent significant differences. $p < 0.05$.

Surface Density (S_V)

No statistical differences were found for the surface density of the granulosa cell layer of the follicles exposed to the different hormones (Fig. 31).

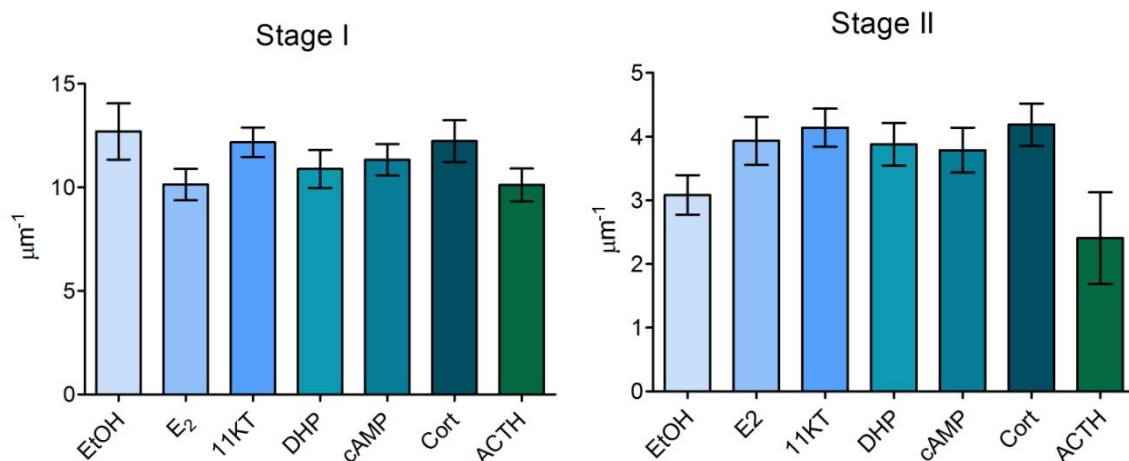


Fig. 31 Surface density of oocytes of stage I and II exposed to the different hormones.

DNA DAMAGE: COMET ASSAY

Validation

The comet assay was established for fish oocytes for the first time. Initially, we validated the new protocol for fish oocytes by using a well known genotoxic agent, the H_2O_2 . Three concentrations were chosen to induce different degree of DNA damage (Fig. 32). The increasing concentrations of H_2O_2 revealed a significant increase of the tail moment (Fig. 33). This demonstrated that the newly established protocol worked and reliably indicated different degrees of DNA damage.

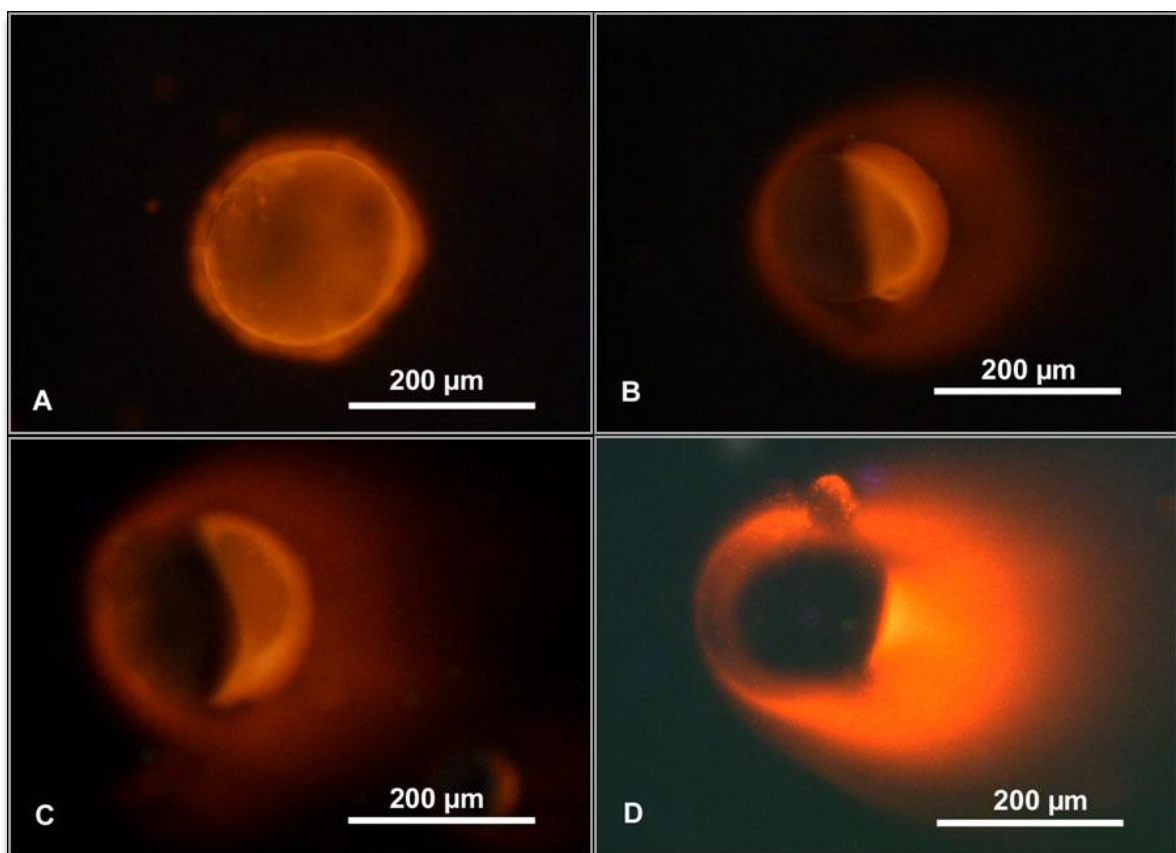


Fig. 32 Examples of comets of the validation procedure. A) Oocyte stage II with no exposure to H_2O_2 ; B) Oocyte stage II with 1 h exposure to 100 μM of H_2O_2 ; C) Oocyte stage II with 1 h exposure to 250 μM of H_2O_2 ; D) Oocyte stage II with 1 h exposure to 500 μM of H_2O_2 .

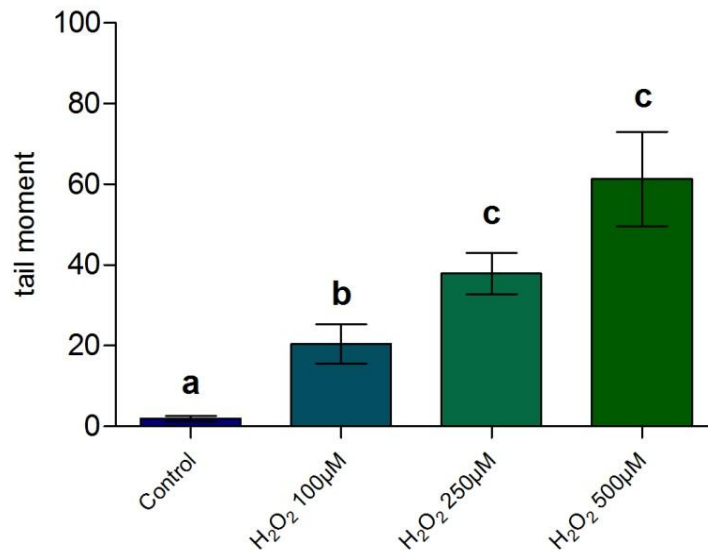


Fig. 33 Validation of Comet assay results. Significant differences ($p < 0.001$) between the control and the exposure to crescent concentrations of H₂O₂.

The used measurement unit was the tail moment, which is the product of the percentage of DNA in the tail with the tail length (µm), values automatically given by the software after manual measurement. The Fig. 34 illustrates how comets are measured by CometScore™.

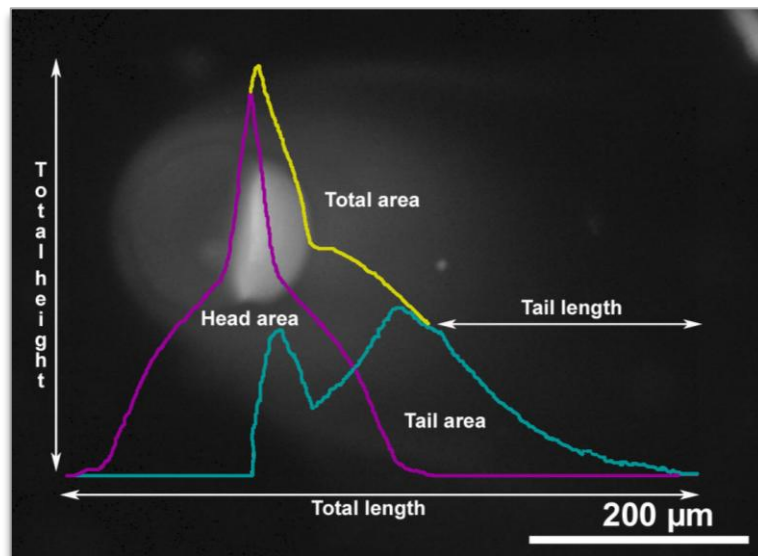


Fig. 34 Example of a comet of a stage II oocyte measured by CometScore™.

Exposure

After validation of the protocol, the oocytes from an exposure experiment with separated oocytes were employed to analyze the induction of DNA damage by the different hormones. The analyses were performed separately for stage I and stage II.

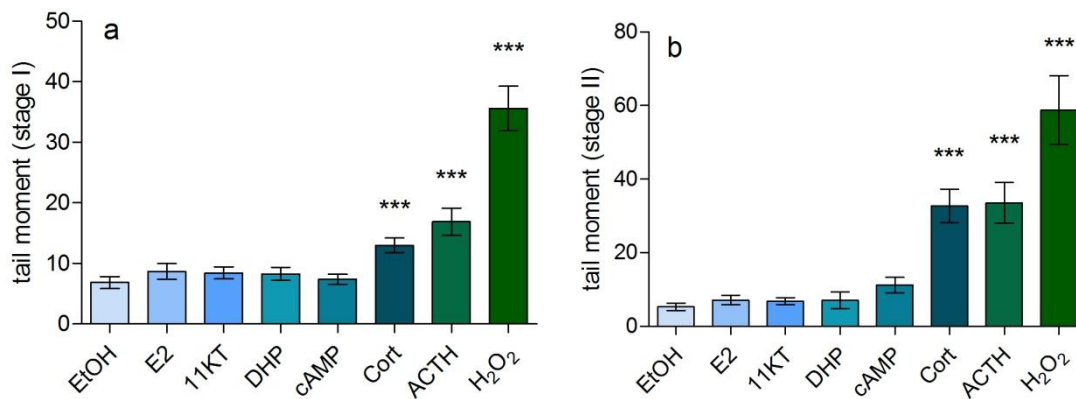


Fig. 35 Comet assay for oocytes exposed to different hormones. a) Stage I; b) Stage II. *** - $p < 0.001$.

The comet assay demonstrated that there was a significant difference between the control and both stress hormones. Cort and ACTH induced a significantly higher DNA damage compared to the control in stage I and II ($p < 0.001$).

DISCUSSION

VIABILITY AND MORPHOLOGICAL CHARACTERISTICS DURING THE *IN VITRO* CULTURE

The first experiments were projected in order to better characterize the oocyte *in vitro* culture. At first, 75% L-15 medium was used since it was the most reported, however observations under the microscope revealed aspects of degeneration especially in vitellogenic oocytes, which were not observed by using 90% L-15 medium. As next, two different approaches were tested to analyze the viability of the oocytes in culture along the time. Generally, the use of several methods is suggested to accurately monitor the viability of the culture, important especially for cryopreservative procedures of oocytes (Fauque et al., 2007; Guan et al., 2008a). The trypan blue test stains cells, if the plasma membrane is disrupted, while the histological screen shows the integrity of the oocytes. However, oocytes are cells with a particular characteristic due to the vitelline envelope, which is formed by the plasma membrane, the zp, and by two layers of follicular cells that surround the oocytes (Abraham et al., 1984). This complex structure may difficult the passage of the trypan blue into the oocyte even when it was unviable, because follicular cells are known as being resistant (Pang and Ge, 2002c) and the zp is a chemical and mechanical barrier for cell protection (Abraham et al., 1984; Modig et al., 2007). On the other hand, in the histological screen only a section of the oocyte is usually observed that opens the possibility to overlook some aspects that may be present in other sections. In our study, both methods indicated viability between 50 - 80% for the oocyte stages I, II, and III, and no major reduction in viability occurred over the culture of 6 days with the exception of stage III oocytes, for that the histological screen revealed a lower viability and first signs of degradation.

To supplement the previous viability tests, a time course was performed for analysis with semi-thin sections fixed in epoxy resins and with TEM. The semi-thin sections revealed in contrast to the other both methods, that already the first signs of degradation were present after 4 days of culture, and that follicular cell layers were frequently damaged. As consequence, thin fragments of ovaries were used instead of separated oocytes in a further time course, which confirmed the results of the previous, but demonstrated a better preservation of follicular cell layers. By further analysis of these fragments with TEM, several characteristics of oocytes at different stages of development were manifested at the ultrastructural level.

At stage I, a beginning cytoplasmic activity was revealed by the formation of organelles (Azevedo, 1984; Azevedo et al., 1984; Zhang et al., 2008) and *Balbany bodies*, which are clusters of mitochondria, ER, Golgi apparatus and MVB (Wallace and Selman, 1990; Tyler and Sumpter, 1996; Guimarães and Quagio-Grassiotto, 2005). A electrodense fibrogranular material appeared near the GV and was suggested to incorporate [³H] uridine as well as being responsible for the generation of organelles such as mitochondria and ribosomes (Azevedo, 1984). Recent studies suggested a role for mitochondria (Reunov et al., 2000; Hara et al., 2005; Zhang et al., 2008; Żelazowska and Kilarski, 2009) in the formation of nuage in the cytoplasm. During the first stage, oocytes became surrounded by a pre-follicle cell layer with a prospection of the microvilli from the oolema through the plasma membrane until the granulosa cells.

At stage II, the formation of CA was observed, which appeared from the periphery until they occupied all ooplasm (Selman et al., 1993). Many organelles, in *Balbany bodies* or dispersed within the cytoplasm, are suggested to be involved in the formation of CA, especially the endoplasmatic reticulum (ER) due to its association to CA as known from other vertebrates (Wallace and Selman, 1990; Terasaki et al., 2001) and Golgi apparatus due to its simultaneous appearance (Wallace and Selman, 1990). CA have been proposed to be involved in the chorion formation (Lubzens et al., 2010) and signal transduction after fertilization (Terasaki and Jaffe, 1991; Terasaki et al., 2001). Granulosa cells at stage II are clearly defined as flattened cells with a large nucleus, which occupies almost all cytoplasm. Furthermore, these cells have large and round mitochondria, which are characteristic for steroidogenic cells (Prince, 2002; Zampolla et al., 2009). Thecal cells are separated from granulosa cells by the basal lamina (Pendergrass and Schroeder, 1976; Abraham et al., 1984; Wallace and Selman, 1990) and the layer is occasionally interrupted by capillary cells, which is an indication of the important function of the uptake of substances from the blood into the follicular layers or vice versa (Abraham et al., 1984; Wallace and Selman, 1990).

Stage III oocytes are characterized by the centripetally accumulation of yolk bodies, which comprise over 80% of the dry weight of the oocyte (Wallace and Selman, 1990). These yolk bodies are frequently associated to a number of organelles as ER, Golgi apparatus or lysosomes that are essential for the metabolism of Vtg and incorporation into the yolk bodies (Wallace and Selman, 1990). The zp is highly perforated by microvilli that are crossing the membranes of granulosa cells. Despite the fact that microvilli are described as being essential for the communication with the oocyte, they do not

participate in the exchange of substances from the blood through the follicular cells. An accumulation of electron-dense granular material appears frequently external to the oocyte attached to the zp, whose nature of this material is not possible to determine by TEM (Selman et al., 1993) and if it is entering or leaving the oocyte. After two days of culture the follicular cells of stage III oocytes contained possible lipid droplets that were absent in the first day of culture.

These general characteristics of oocytes at different stages and during the serum-free *in vitro* culture along several days implied that the oocyte-follicle complexes displayed ultrastructural aspects as it is known from the literature and therefore showed a normal development. However, at day 4 of the oocyte culture first signs of degeneration were observed in oocytes from stage II and III, namely the deformation of ER, vacuolization, loss of follicle cells and a general degradation of the cytoplasm.

In some other studies, oocytes were cultured for 6 days (Pang and Ge, 2002a), while also some cultures are described with 10, 15, 18 or 20 days exposure time (Kortner and Arukwe, 2007; Lokman et al., 2007; Miura et al., 2007; Kortner et al., 2008). However, in these studies the medium was complemented by serum, nutrients, hormones or other factors and the viability was not thoroughly assessed. In a recent study, the viability of *in vitro* cultures of early stage oocytes (stage I and II) was analyzed using trypan blue staining and it was concluded that the viability of ovarian follicle complexes decreased after a culture time of 24 hours (Tsai et al., 2010).

In our study, we established the baseline for a serum-free *in vitro* culture of zebrafish oocytes using different methodologies to characterize the viability of oocytes in the used culture conditions. Our results showed that normally used methods detected a decrease of viability after 6 days, but that TEM already demonstrated cell degenerations after 4 days. As a consequence, the exposure experiment was performed with an oocyte culture during 2 days, where a good rate of viability could be expected according to the applied methods.

IMMUNOHISTOCHEMISTRY OPTIMIZATION

The connection between the follicle cells and oocytes is further characterized by the presence of tight junctions (TJ) and adherens junctions (AJ). In zebrafish tight junctions (TJ) are present between follicular cells but not between follicular cells and the oocyte (Cerdà et al., 1999; Clelland and Kelly, 2010). In contrast, adherens junctions have been identified between the oocyte and the follicular layer in zebrafish, which are formed by cadherin-catenin complexes (Cerdà et al., 1999). In the present work, conditions were

established that demonstrated a successful binding of a monoclonal mouse anti-human e-cadherin antibody to the follicle layer of zebrafish oocytes. However, the samples derived from the *in vitro* culture showed a less clear binding for unknown reasons. These difficulties may be related to the chosen procedures, but could not be identified. The antibody marked E-cadherin not only in the zebrafish intestine, but also in fresh gonads. The two days of culture revealed good quality of the oocyte and the follicular cells in TEM. The HistoGel™ is adequate and broadly used in IHC (Torres-Vélez et al., 2003; Monaco et al., 2011). Two hypotheses are proposed to explain these different binding affinities. First, the shrinking effect of paraffin (Baird, 1936) in the tissue was quite visible in histology sections and maybe the use of the entire gonad permits a higher protection and preservation of the follicular layers from shrinkage, especially of the more inner oocytes. Secondly, in the culture, follicular cells starts to proliferate and the adherens junctions are therefore less developed.

ULTRASTRUCTURAL CHARACTERIZATION OF OOCYTES EXPOSED TO THE DIFFERENT HORMONES

The normal histology procedures using Goldner staining did not detect any morphological changes in the oocytes exposed to the hormones. The different oocyte stages I, II, III and IV in all exposure groups displayed a normal appearance as in the control group, and on a qualitative level no major alterations were present in relation to the presence of perinuclei, of CA, of yolk bodies or the formation of the follicle cell layers.

Further analysis were performed using TEM to reveal potential, qualitative alterations on the ultrastructural level of the oocytes of stage I and II in response to the hormonal treatment. Thereby, several parameters were evaluated on the ultrastructure of the periphery (the follicle cell layers) and of the inside of the oocytes (ooplasm). In stage I, the majority of the hormones - E₂, 11KT, cAMP, Cort and ACTH - did not influence the morphology of the follicle cell layers. However, DHP seemed to have an influence on the premature appearance of the granulosa layer in the stage I. As a response to the hormonal treatment, a second follicular cell layer appeared in the DHP exposed oocytes of stage I in 70% of the analyzed individuals, while this was the case only in 8.3% of the individuals of the control group. Therefore, it is possible that DHP has the capacity to stimulate the growth of premature granulosa cells and may be involved in the early formation of the follicular cell layers in stage I. In contrast, cAMP revealed a high

percentage of cells that did not show the presence of thecal cells (66.7%), and may therefore delay the occurrence of the follicular cells.

Accordingly to the effects on the follicular cell layers, the majority of the hormones did not interfere with the maturation of the ooplasm at stage I. Only the exposure to Cort revealed an effective and visible action on the inside of the stage I oocytes. A high proliferation of the sER was observed in 66.7% of the individuals, while in the control group this effect was not observed. Therefore, the observed induction of the ER proliferation may indicate an increased processing or production of lipids and generally an advancing in the maturation, since the ooplasm in the following stages is richer in the presence of organelles. The second finding as a response to the Cort treatment on stage I oocytes supports this hypothesis. An apparent more reticulated GV was observed in the Cort exposed stage I oocytes, which is a sign of an increased nuclear activity and occurs in fact, when oocytes are passing to the next stage (Wallace and Selman, 1990; Selman et al., 1993).

On stage II oocytes, the exposure to different hormones resulted in several alterations on the ultrastructural level, especially on the ooplasm. Some qualitative effects were found for E₂, cAMP, Cort and ACTH, which differed from the appearance at the control group. The exposure of stage II oocytes to E₂ revealed a higher production of ER around the CA. In 80% of the individuals an increased formation of ER was detected around the CA, while in the control group only in 50% of the individuals a moderate level was present. This result is consistent with previous studies that support an association of FSH and E₂ with the accumulation of CA before the production of Vtg in the liver (Khoo, 1979; Campbell et al., 2006). Here, we present further evidence that E₂ may be involved in the formation of CA by the increase of organelles observed in TEM, namely the ER around the CA.

Another alteration was observed in stage II oocytes exposed to cAMP, where the sER was highly proliferated in the ooplasm (100% of individuals). The sER is mainly involved in the production of lipids and steroids. However, the production of steroids is mainly localized to the follicle cell layers, and the oocyte does not possess StAR enzyme activity at stage II (Ings and Van der Kraak, 2006; Clelland and Peng, 2009). Therefore, it is very likely that the cAMP induced sER formation in stage II oocytes is related to the production of lipids. This hypothesis is supported by the study of Campbell (2006), which suggested a role for FSH not only in the CA, but also in the lipid droplets accumulation. Since cAMP mimics the actions of GTH, this result may indicate that GTHs may be involved in the production of lipids or glycolipids. This result is also interesting, since the early stages of the oocyte development are regarded to be GTH-independent. Here, we provide further

evidence for a role of GTH in the preparation of oocytes at stage II for the lipid production or accumulation. Further research is needed to confirm this observation and to discriminate, whether the effects will be due to FSH or LH actions.

The exposure to stress hormones also has an obvious action on the ultrastructure of stage II oocytes. The Cort exposure led to the high proliferation of apparent rER especially in the form of annulate lamellae. The annulate lamellae is a membranous structure morphologically similar to the nuclear envelope and consists of roughly parallel membranes, which appear as single profiles, stack in a parallel array or even in a concentric arrangement. Annulate lamellae are frequently continuous with the rER (Wallace and Selman, 1990) and appear before the vitellogenesis (Wallace and Selman, 1990; Selman et al., 1993). In our qualitative analysis, 100% of the individuals were characterized by very high proliferation of rER in the ooplasm of stage II, while this effect was only observed in 33% of the individuals of the control group. Therefore, we assume that the apparent induction of the rER formation is related to a higher processing or production of proteins, which could indicate generally an advancement of the maturation of stage II oocytes. From studies in amphibians, it is well known that glucocorticoids accelerate the development of amphibian larvae due to the synergistic action of glucocorticoids and thyroid hormones, which is generally recognised as an adaptation to altering environmental conditions, e.g. desiccating temporary pools (Denver, 2009). If a similar scenario would be true in fish species, Cort release in response to stressful aquaculture holding conditions could be involved in the faster maturation of fish species in aquaculture. Early puberty of fish is a common problem in aquaculture production, since fish then suffer from reduced growth rates (Taranger et al., 2010). Further investigations are needed to analyse this speculative association.

Moreover, the exposure of stage II oocytes to Cort increased the diameter of the zp layer (100%). In accordance to the other Cort induced effects, the thickening of the zp could be another sign of ongoing maturation of the oocytes. In fact, it is known that the zp increases in size until the maturation phase, where it retracts for ovulation (Gosden et al., 1997). Another possibility could be that the thickening of the zp is associated with cellular atresia, however existing results are not that clear. On one hand this phenomenon has been reported as a possible sign of *atresia* (Henderson et al., 1987), which is the degeneration of the follicle walls and then the oocyte (Miranda et al., 1999). On the other hand, it has been demonstrated that even in *atretic* oocytes the zp size remains (Devine et al., 2000; Høst et al., 2002).

Despite the fact that stress hormones are known to suppress the gonadal activity (Kiess and Gallaher, 1998; Mommsen et al., 1999; Fuzzen et al., 2011), they may also play a different role in the reproduction as it is suggested in previous studies related with ovulation (Bry, 1985), where stress hormones acted as inducers of the oocyte development. The presented results supported this last suggestion, especially since Cort targeted the formation of the ER, which has important roles on calcium intracellular signalling that is essential for the activation of target proteins as protein kinases and phosphatases and frequently related to cell growth and differentiation (Terasaki et al., 2001; Alberts et al., 2002a; Cooper and Hausman, 2007b).

The second stress hormone used in this study, ACTH, had a severe impact on the development of oocytes at stage II and the action of ACTH was the most surprising in this study. As a response to ACTH, a degradation of stage II oocytes was widespread and decreased the viability in 83.3% of the individuals. As signs of a decreased viability, the degradation of mitochondrias and CA, the distortion of annulate lamellae, the development of numerous vesicles and of phagocytic features, and a distorted and fragmented zp were frequently observed on the ultrastructural level. These events were characterized in previous studies as typical alterations in atretic oocytes (Henderson et al., 1987; Miranda et al., 1999).

It is inevitable to relate the *atresia* with the programmed cell death that it is known to be frequent in the ovary of vertebrates (Kaipia and Hsueh, 1997; Baum et al., 2005; Krysko et al., 2008). In fact, prior studies describe “Ovarian follicular degeneration or *atresia* is a hormonally controlled apoptotic process, whereby degenerating follicles are eliminated in a coordinated fashion” (Hsueh et al., 1994; Kaipia and Hsueh, 1997) and many studies have been developed in order to prove this relationship between apoptosis and hormonal activity (Hsueh et al., 1994; Janz and Van Der Kraak, 1997; Kaipia and Hsueh, 1997; Chun and Hsueh, 1998; Kiess and Gallaher, 1998; Høst et al., 2002).

Interestingly, the receptor for ACTH was only recently discovered in gonads of zebrafish, while it is known to be localized into the adrenal cells. The function of this receptor was not described yet, but it was shown already that a crosstalk of this stress hormone existed with the reproductive system, since ACTH repressed the hCG-induced E₂ production (Alsop et al., 2009). Here, we demonstrated for the first time, a direct function of the ACTH receptor in oocytes of stage II by the finding of an increased rate of organelle degradation and apoptosis. Therefore, it is possible to speculate that ACTH not only acts on the adrenal gland to mediate a stress response via stimulation of glucocorticoid production, but that ACTH has a direct action on the fish ovary to promote gonadal apoptosis.

However, it has to be emphasized that only a qualitative analysis was performed in the course of our study regarding the ultrastructural alterations in response to different hormones. A qualitative analysis in combination with a contingency analysis is suitable to indicate whether and which potential alterations may occur in the exposed oocytes compared to the control oocytes. Some random effects cannot be excluded since in TEM only few oocytes of each of the 6 individuals were analyzed. Therefore, the joint consideration of stage Ia and Ib, and stage IIa and IIb as stage I and II, respectively, could be a source of misinterpretation if randomly a different percentage of the sub-stages were evaluated. As characterized above, the sub-stages already represent major differences in the oocyte maturation. These qualitative results were necessary to guide future research and taking this into account, our results would profit from a quantification of the observed morphological alterations as well as from a determination of the effect level. More subtle alterations may only be detectable by a profound quantification strategy and some of the interesting findings should be complemented by quantitative methods as stereology, confocal microscopy or molecular biology.

STEREOLOGY

A stereological approach was chosen to get insights into the interference of some of the hormones with the formation of the follicle cell layers. Two different parameters were analyzed, namely the nuclear volume and the surface density that served as indications for cell proliferation or increase in cell size, respectively. The oocytes at stage I, IIa, IIb and III changed the nuclear volume in response to the exposure to 11KT, DHP, cAMP and Cort to different extents dependent of the oocyte stage (compare Fig. 29). The main effect was observed for all hormones at stage IIb, while on stage I only 11KT and on stage III only Cort affected the nuclear volume. Surprisingly, these hormones decreased the nuclear volume of the granulosa cells in all analyzed oocyte stages.

For interpretation of these results of V_V obtained in stereology, it is important to refer to the cell cycle. As it is known, the eukaryotic cell cycle comprises four discrete phases: the G1 (gap1) phase, which corresponds to the interval between mitosis and DNA replication; the S phase (synthesis), where the DNA replication occur; the following G2 (gap2) phase, where the cell progressively grows and proteins are synthesized for the last phase; the M phase, which corresponds to mitosis and ends with cytokinesis (Alberts et al., 2002b; Cooper and Hausman, 2007a). However, cells are not always dividing. In the quiescent G0 phase, the cells remain metabolic active, but are not proliferating until extracellular signalling. Due to this information, previous work tried to associate the nuclear and cell

volume with the course of the cell cycle (Van'T Hof and Sparrow, 1963; Cavalier-Smith, 1978; Steen and Lindmo, 1978; Fidorra et al., 1981). These studies suggested that during the G1 phase the nuclear volume does not change until the onset of DNA synthesis, which corresponded to the decondensation of chromatin and the preparation of the S phase in that a nucleus increase is verifiable. Then, at the beginning of the G2 phase the nucleus reaches its maximum volume due to DNA replication and decreases again with the start of the mitosis (Steen and Lindmo, 1978; Fidorra et al., 1981).

Analyzing the present results of nuclear volumes, 11KT, DHP, cAMP and Cort significantly decreased the nuclear volume compared with the respective controls. Taking as base the work of Fidorra (1981), the decrease of nuclear volume suggested that the granulosa cells were predominantly in the G0 or G1 phase in that the nuclear volume is the lowest. One possible interpretation of this result could be that the different hormones induced cellular proliferation in the granulosa cell layer and that all cells were back in the G0/G1 phase after finishing mitosis. This could be true if the interphase of the granulosa cell cycle lasts long and therefore all the cells were in this phase at our sampling time after 48h exposure. Another interpretation could be that the hormones caused the opposite effects, namely inhibiting the cell proliferation and forced the cells into quiescence (G0 phase). Not many studies were made in order to verify nuclear volumes of granulosa cells. A work with human granulosa cells observed a small variation (8.5%) between nuclear volume of granulosa cells of mature follicles, however in this study only matured follicles of unknown LH concentration exposure were used (Dhar et al., 1996). In the normal course of oocyte maturation, the nuclear volume of the granulosa cells increased significantly from stage I until IIb, and decreased in volume at stage III and IV (see results, Fig. 30). In this light, the hormonal induced decrease in nuclear volume could be related to the normal decrease in nuclear volume at the later oocyte stages. This could imply that the observed hormonal signalling led to a reduced granulosa cell proliferation, which at this stage could chaperon the switch from the FSH directed growth phase of the oocytes to the LH induced final oocyte maturation.

COMET ASSAY

The comet assay, or single-cell electrophoresis, is a widely used assay that detects single or double-strand breaks and therewith genotoxic effects of compounds or apoptosis (Liao et al., 2009). The comet assay was used so far with a huge variety of cells, including oocytes of mammalian species, but was here employed for the first time with teleost oocytes. Therefore, the existing protocols had to be adapted and after a thorough analysis of assay conditions, a final protocol was successfully validated with different concentrations of a known genotoxic agent (Fig. 33).

The exposure of oocytes at stage I and II revealed that only the both stress hormones, Cort and ACTH, induced a significant increase in the tail moments in both oocyte stages (Fig. 35). Probably both stress hormones act on their corresponding receptors in the ovaries and induce directly apoptosis, which is characterized by the appearance of first single-strand breaks, and in advanced stages double-strand breaks. The results achieved in the comet assays are consistent with the qualitative results obtained from the ultrastructural observations, at least for the action of ACTH. Here, the induction of apoptosis in the comet assay by ACTH corresponded very well with the induction of degradation of organelles as observed in the TEM. ACTH is normally studied in relation with its action on the adrenal glands, but here it is for the first time demonstrated that it may have a different function. Further tests, like mRNA expression of apoptotic molecular signalizations could be applied to identify if this DNA degradation resulted from an apoptosis mechanism.

In contrast, Cort increased the intracellular activity as seen by the proliferation of rER in the TEM, while degradation was not observed. One explanation for this divergence could be that the best sets of oocytes were selected for the qualitative analysis with ultrathin sections for TEM and therewith apoptotic cells were less common. However, the same should be true for the sections with ACTH and no such effects could be seen there. Another explanation could be that Cort has several targets and could sequentially stimulate the proliferation of rER and induction of apoptosis, especially since the exposure to Cort lasted 48h at a high concentration. In previous studies with different cell lines as blood cells, neuronal or adrenal cells, it was proposed that glucocorticoids act as inducers of apoptosis (Kiess and Gallaher, 1998). However, there is no further *in vitro* study that analyzed the effect of Cort on oocyte at the stage I and II.

In protogyne hermaphroditic fish species like zebrafish, this discussion about apoptosis has a special importance because of its particular characteristic of sex-reversal. In zebrafish, it was proven that apoptosis is a general mechanism that is involved in the sexual differentiation and that apoptosis may occur either in follicular cells or in the oocyte with the aim of the transformation from the ovaries into testis (Orban et al., 2009). In our study, the sexual steroids did not induce apoptosis. However, it is possible that sexual steroids may play a role as survival factors, or so called anti-apoptotic agents.

In vitro modeling of the oocyte development in zebrafish (*Danio rerio*): the role of hormones in maturation, apoptosis and sex-reversal of oocytes at different development stages

CONCLUSIONS

The presented master dissertation aimed to analyze the role of hormones on the maturation of oocytes at different developmental stages and on the induction of apoptosis, a process that is important for the sexual differentiation of zebrafish. At first, serum-free *in vitro* culture conditions for fish oocytes were evaluated with different methodologies to learn about the viability of oocytes during the culture and to define the maximal exposure time. Interestingly, the TEM was the most sensitive methodology and revealed a degradation of cell organelles at 4 days of culture in stage II and III oocytes, which was not visible with the other methodologies (standard histology, semi-thin sections, and trypan blue staining). Herewith, we investigated the basic preconditions of serum-free *in vitro* culture of follicle-enclosed oocytes, which may serve as a foundation for future investigations.

In the exposure of oocytes to different hormones, an interaction of E₂, cAMP and Cort was observed on the maturation of the ooplasm by inducing the formation of organelles at stage II oocytes (ER around CA, sER and rER, respectively). Generally, a stimulating role was concluded for these hormones and speculated that E₂ could be involved in the formation of CA, and cAMP in the accumulation of lipids.

The major finding in this study was the characterization of the action of both stress hormones on oocytes at stage I and II. While Cort seemed to have a stimulating role on oocyte maturation, the exposure to ACTH caused cellular degradation and the induction of apoptosis. In the case of ACTH, this action is supported by the results of the TEM and comet assay. For the first time, it has been demonstrated that ACTH not only acts on the adrenal gland, but also has a direct effect on the ovary. With this, first evidence is presented for a possible function of the ACTH specific receptor in the zebrafish gonad (Alsop et al., 2009).

Furthermore, an interaction of some hormones was observed with the formation of the follicular cell layer of the oocytes. The exposure to DHP on stage I oocytes suggested a stimulating role on the proliferation of granulosa cells. 11KT, DHP, cAMP and Cort reduced the nuclear volume of granulosa cells in response to the exposure at different oocyte stages, however, the interpretation of this finding need a further complementation by molecular studies.

In a word, this work contributed to the knowledge of fish endocrinology and showed interesting roles for hormones on the maturation of oocytes and the formation of the follicular cell layer.

Future Ideas

Future research can be orientated in order to complement these present results.

Starting with the culture conditions, a comparison of the used *in vitro* culture conditions with oocytes cultured in the presence of serum would be interesting. The results of this proposed experiment will also support the already known idea that hormones and factors are essential to gonad survival (Janz and Van Der Kraak, 1997; Kiess and Gallaher, 1998; Van Der Kraak and Lister, 2011) and several factors could be tested for the potential to extend the culture time.

Another improvement to the *in vitro* culture could be done in order to do culture of follicular cells without oocyte (Pang and Ge, 2002c). The exposure of hormones may reveal some insights about paracrine activity between follicular cells, between follicular layers and the oocyte and about the uptake of substances. This last work proposal lead us to another important question about the exact localization of hormone receptors. Several studies show us the presence of hormones receptors in the gonad (So et al., 2005; Clelland and Peng, 2009). However do not refer the precise localization of those receptors: oocyte or follicular cells? IHC should also be improved and extended to other antibodies in order to better understand the formation of communication structures (as Gap, Tight or Adherens junctions) and the influence of hormones in this process.

To better support the present work, molecular techniques are also indispensable to complement the morphological changes observed in this work and to get insights into molecular mechanisms. For example, the analysis of apoptotic genes would be interesting to confirm the indication of apoptosis by ACTH.

Values of the study

Fishes are remarkable in their diversity, not only in the abundance of species, but also in aspects of their morphology, ecology, genetics, physiological functioning and behaviour. These characteristics make them a very interesting group for various research lines. Furthermore, biodiversity is also important in other perspectives: ecological, cultural and economically (Volff, 2004). Attending to the economical area, the aquaculture is an emergent business, where science still can contribute to improve and promote its progress. The natural resources are declining, including natural fish stocks. For this reason, aquaculture is a viable alternative, which may contribute to the supply of food to

mankind. However great improvement is still needed regarding some ecological and economical aspects.

Great efforts have been made in recent years to improve the quality of fish eggs and to increase the rates of viability. These improvement of the fish egg quality can be manipulated by optimizing diets, having a genetic selection of progenitors, controlling of environmental factors but also by understanding and manipulating endocrine regulation of oocyte development (Brooks et al., 1997; Lubzens et al., 2010). Still major achievements are needed for the production of the so called “high-quality eggs” and for a successful broodstock management, which still depends for many species on the catchment of adult spawner. Knowledge about the development of early oocyte stages and the formation of follicle cells could be helpful to unravel the mechanisms that are responsible for egg quality.

In vitro modeling of the oocyte development in zebrafish (*Danio rerio*): the role of hormones in maturation, apoptosis and sex-reversal of oocytes at different development stages

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