universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Cryofixed Artemia: A New Approach To Better Ultrastructure“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2016 / Vienna 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 878
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Verhaltens-, Neuro- und Kognitionsbiologie
Betreut von / Supervisor:	Ao. Univ.-Prof. i. R. Dr. Waltraud Klepal

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1. Introduction

1.1. Basic biology of *Artemia franciscana* (Kellogg, 1906)

The brine shrimp *Artemia franciscana* (Crustacea: Branchiopoda) occurs in hypersaline lakes all over the world. This crustacean has a highly efficient osmoregulation system, which enables it to adapt to such extreme habitats. Furthermore these animals are able to switch between two different reproductive ways to maintain their population. Under suitable environmental conditions, freeswimming nauplii are released from the female's ovisac (ovoviviparity) (Clegg and Trotman, 2002). As soon as the environmental circumstances become unfavorable, the females produce metabolically inactive eggs, namely cysts, which undergo diapause in a late gastrula stage (Clegg 2002). Throughout the seasonal drying-out period of the lake, the cysts are able to survive on the bottom of the lake without hydration. Rehydration due to rain is necessary to animate the cells for continuation of their development (for review see Browne et al., 1991).

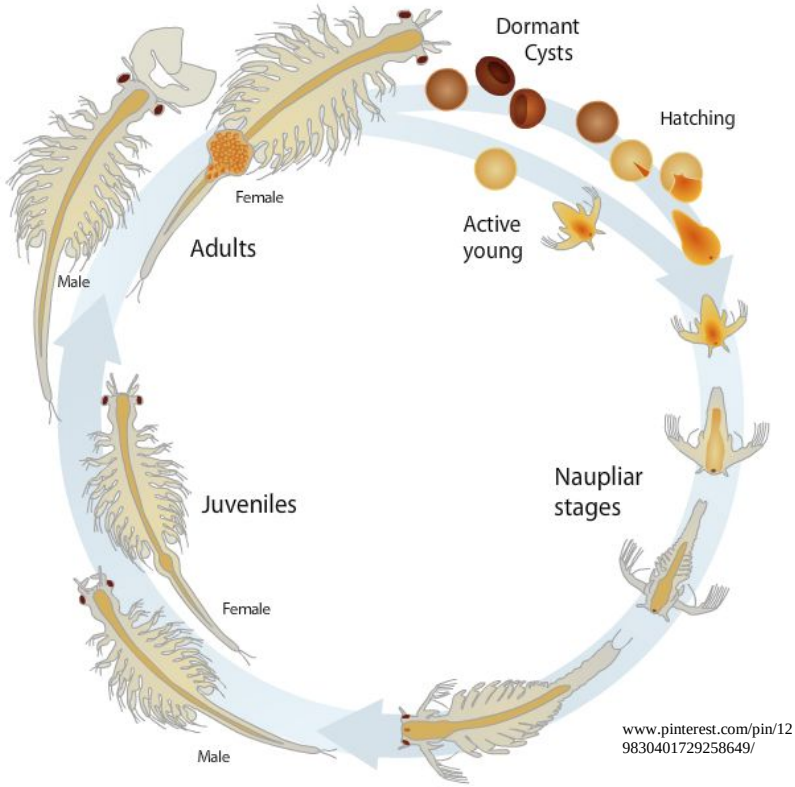

Fig. 1: Life Cycle of *Artemia sp*.

The cells of the encysted embryo are protected by a four-layered eggshell. The outermost hydrochloride-soluble substance, secreted by the maternal shell glands, is a mechanical barrier. Additionally three layers of cuticle strengthened by chitin are made by the blastocysts of the embryonic tissue, consisting of an outer and an inner cuticle membrane and a fibrous layer in between. All three layers are impermeable for non-volatile solutes and represent a functional biochemical barrier. This structural composition of the egg shell is the best prerequisite to survive harsh environmental cues without water (Abatzopoulos et al. 2002; Dai et al. 2011).

***Artemia franciscana*: a model for adaptive biochemical and physical mechanisms**

Stopping metabolic activity to survive harsh environmental circumstances has also been found in many other invertebrate taxa such as rotifers, nematodes and tardigrades (Watanabe et al. 2004). Many studies focus on this unusual state of metabolic arrest (MacRae 2016). Since many decades scientists also investigate the underlying biochemical mechanisms in *A. franciscana*. They found in the state of desiccation (anhydrobiosis) large concentrations of polyhydroxy compounds, like the disaccharide trehalose, which is thought to play a crucial role in biochemical adaption (Criel and Macrae, 2002).

Besides, there is tremendous economic interest in nauplii, larva of the brine shrimp, as nutritious food for fish breeding (Sorgeloos et al., 2001). Especially the cysts of *Artemia salina* and *Artemia franciscana* are two of the most sold species all over the world. Feeding with freshly hatched nauplii is essential for the successful rearing of some fish species.

The flexibility of *A. franciscana* in reproduction and the post diapause development have fascinated many scientists till now (Browne & Wanigasekera 2000; Sorgeloos et al. 2001). Living in fast changing habitat, these encysted embryos are highly resistant to extremes of environmental stress, while the larva and the adults are some of the best osmoregulators (Clegg and Trotman, 2002). Therefore, many biological and applied studies have chosen *A. franciscana* as a model for these rare adaptive capabilities (Hengherr et al. 2011).

Electron microscopy (EM) has been applied extensively for the investigation of the anatomy and morphology of the motile stage of this animal. Previous studies included reproductive organs,

fertilization, vitellogenesis and oogenesis, but also the preparation of the cyst and its content (Criel, 1992; Bartolomaeus et al., 2009)

1.2. Sample preparation for transmission electron microscopy

By using transmission electron microscopy (TEM) visualization of the ultrastructure of morphological issues becomes possible. As precondition samples have to be prepared adequately to preserve the natural state of the cells and the ultrastructure of their cellular content, like membrane- and non-membrane bound organelles.

1.2.1. Chemical preparation at room temperature

In the history of the use of EM in life sciences the chemical fixation and processing at room temperature became the standard procedure (Thavarajah et al. 2012). A typical protocol for TEM comprises: primary fixation (e.g. glutaraldehyde) and post fixation with osmium tetroxide. Notably, a buffer is required as vehicle for the fixatives. Chemical fixation is followed by dehydration e.g. with a series of ethanol or acetone, subsequent infiltration and embedding in resin.

Chemical preparation takes a relatively long time and the application of organic solvents has a strong impact on ultrastructure. The most obvious artifacts are: rearrangement or leaching of cell components, like proteins and lipids, occur usually and chemical preparation takes a relatively long time. The longer the primary fixative needs to penetrate into the tissue, the more changes by autolysis may occur. Consequently, the natural state of the tissue becomes distorted during processing.

Moreover, the success in chemical preparation depends on the nature of the specimen: Cell walls and cuticle formations reinforced by resistant biological material, like cellulose or chitin, hinder the penetration of the fixatives. Usually these difficulties are met by smaller samples and/or longer fixation times. They are also accompanied by additional destruction of the tissue.

Studying the development of the hard-shelled cysts of *Artemia franciscana* by using chemical fixation will be difficult. The intrusion of fixatives and resins into intact specimens protected by chitinous cuticle can be hardly managed. For the study of cysts, decapsulation or nicking of the eggshell to facilitate the entry of chemicals has so far been the method of choice (Dai et al. 2011). This causes not only to the destruction of the cyst, but also to artifacts in the embryonic tissues.

1.2.2. Cryopreparation

Freezing the tissue and subsequent fixation and dehydration at low temperatures brings several advantages:

i) The biological material is immediately immobilized next to its natural state
ii) The rearrangement of cell material is strongly reduced
iii) The use of buffer is avoided
iv) Leaching of biological material during dehydration and resin infiltration can be significantly reduced

High pressure freezing (HPF) became a welcome alternative to chemical fixation during the last decades. Immediate immobilization of the cell, by applying in liquid nitrogen at 2000 bar contributes to almost native cell preservation and prevents major shrinking artifacts (Kaech & Ziegler 2014).

The subsequent freeze substitution (FS) is critical for dehydration and fixation of the biological sample (Hippe-Sanwald 1993). The frozen aqueous phase is dissolved in an organic solvent, for example acetone, at low temperatures at about -80 °C to -90 °C. Consequently processing should be as short as possible to avoid changes in the cell and tissue ultrastructure. Different protocols are applied according to their suitability for the specimen type. Commonly FS machines are used, which allow automatized running of exact time/temperature schedules. The possibility to add chemical cross linkers at low temperatures reduces the destruction of the biological sample even further.

Agitation of the specimen during FS should facilitate and accelerate the exchange with the selected medium (Mcdonald & Webb 2011). Therefore, we apply the prototype of an agitation module that was developed in our EM lab.

A. franciscana have not been prepared by cryopreparation so far. However, based on previous publications on species other than brine shrimps one can anticipate major improvements if compared with conventional chemical processing at room temperature. Interactions between different cell organelles are clearly detectable, lipid membranes become smooth and clearly visible. Ribosomes and glycogen appear organized in cellular clusters rather than evenly redistributed in the cytoplasm (Reipert et al. 2004; Korogod et al. 2015) . The approach presented here tests a new way to overcome tough membranes of the animal and its eggs. Inevitably, this will give us with new insights and hypotheses inspired by cryopreserved morphology and its ultrastructure.

1.3. Aims of the study

The studies of cryoimmobilized brine shrimp tissues had two main targets:

1. Improvement of the cryopreparation techniques HPF and FS.

This task included establishing protocols and handling skills for improvements of the sample preparation in comparison to conventional chemical preparation at room temperature (RT). Within the framework of the Prize 2015 project for prototype development (P1404894), funded by the Austrian Science Service, a novel agitation module for acceleration of the automatized FS had to be tested and appropriate temperature/ time schedules had to be developed.

2. Application of cryopreparation (point1) to the nauplia and cysts of *Artemia franciscana* which differ in their resistance to chemical substitution processes.

Tissues of the female reproductive organs were chosen: i) dissected ovisacs, including cysts at different developmental stages, and ii) rehydrated cysts with developed embryonal cuticle. The *A. franciscana* strain Vinh Chau (Vietnam) was chosen for culture and investigation since its comparably small cyst size (200 –230 µm in diameter) suits the freezing conditions better than larger cyst diameter of other species.

The morphology und ultrastructure of resin embedded samples had to be studied, and quality parameters of the preparations had to be assessed (fixation quality, completeness of both FS and the resin infiltration, degree of extraction of biological material, etc.). Attention had to be paid to the organization of small, non- membrane bound cellular constituents such as glycogen and ribosomes, since they are known to be prone to redistribution and losses under conventional processing at room temperature. Aspects of the *A. franciscana* morphological and developmental ultrastructure, which gained most by cryopreparation, had to be identified and discussed in the context of cell biology.

2. Material and Methods

2.1. Culturing *Artemia franciscana*

Dried cysts of *A. franciscana*, Vinh Chau (Vietnam) and TUZ (Turkey) strains were provided by Prof. van Stappen, Laboratory of Aquaculture & Artemia Reference Center of the University of Gent. The larvae were reared under laboratory conditions to the adult stage as follows:

350 g of sea salt for aqua culturing ´Reef crystals´ (Aquariumsystems, Switzerland) were dissolved in 10 l doubled distilled water (ddH_2O) and applied for cultivation. This comply the salinity of sea water, measured in parts per thousand (ppt) with a refractometer (Aqua medic GmbH, Bissendorf, Germany).

Hatching of nauplii from the cysts occurred in duran© vessels, 6 cm high and 10cm in diameter, filled with 200 ml water of 35 ppt salinity. After 2 days, nauplii were selected into culture medium bottles with a capacity of 500 ml saltwater of the same salinity. To provide stable rearing conditions, the number of individuals was limited to 15 to 20 nauplii per bottle. During rearing the salinity of the water vary in a range between 35 to 40 ppt mainly because of evaporation. For compensation pure ddH_2O was added.

16 h of Illumination per day ensured a proper hatching rate (Vanhaecke & Sorgeloos 1982) LED arrays (white light, 2x 40 cm x 40 cm, 30cm distance from culture bottles) were used as light source to avoid uncontrolled heating of the water (Fig. 2). The water temperature varied between 22 to 28 °C, depending on summer or winter months.

For feeding, algae of the two strains, *Rhodomonas* sp. and *Isochrysis* sp. (courtesy of Dr. Daniel Abed Navadi, Haus des Meeres Vienna) were added daily to the culture bottles. Algae, delivered in 5 liter plastic bottles, were also placed under the LED arrays, to ensure their survival (Fig. 2). For our studies, oviparous eggs, namely cysts, freshly released from the adult females ovisacs, and rehydrated cysts were used. Additionally, the females ovisacs, including developing cysts, were dissected and already hatched nauplii of different stages were prepared.

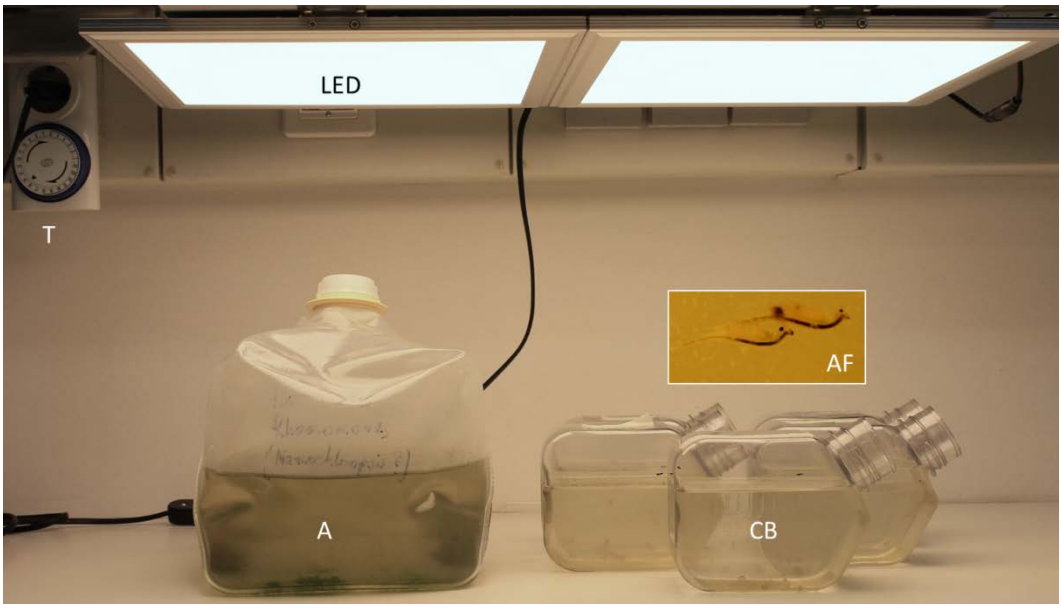

Fig. 2: *Artemia franciscana* culture. Culture bottles (CB) with *Artemia franciscana* (AF) and algae (A) lighted by two LED arrays (LED), switched automatically by using a timer (T)

2.2. Chemical sample preparation at room temperature supported by microwave radiation

2.2.1. Fixation

4 % PFA was dissolved in PHEM Buffer (600 mM PIPES, 250mM HEPES, 100 mM EGTA and 20mM, $MgCl_2$; pH 7, 4) on a stirring/ heating plate at 70 °C. Additionally 5% sucrose was added.

Adult females were selected, put on crashed ice to reduce the animal's movements, and decapitated with a micro-scissor. The thorax and the abdomen was removed stepwise until the ovisac remained. This procedure took less than 2 min for each animal. Cysts were rehydrated with salt water (35 ppt) for at least 12 h before fixation. They were collected with a pipette (20 µl; Gilson) and shortly dried on filter paper. Moreover, nauplii at different developmental stages were decapitated.

Immediately afterwards, both, the eggs and the dissected ovisacs, were immersed in 3 ml fixative contained in 3ml glass vials with conical bottom (V- vials©; EMS, US). Either 10 to 20 cysts or two ovisacs were added per vial.

To support fixation the specimen was placed in a temperature-controlled laboratory microwave oven, PELCO BioWave® Pro (Ted Pella, Inc, US). A ´Steady State´ tray filled with a circulating water load was inserted to ensure even distribution of the microwaves (MW) (Zechmann and Zellnig 2009). Vials with samples were placed in the middle of this tray and exposed to MWs for 30 min at 200 W. A temperature sensor was inserted into the fixative and the temperature limit was set at 36 °C. After MW exposure, the vial with the specimen was placed into a fridge at 4 °C for fixation overnight. Processing was continued by 3 times washing with the PHEM buffer for 5, 10 and 15 min. Samples were post-fixed for 1h under agitation with 1% osmium tetroxide (OsO_4), followed by 3 washing steps with PHEM buffer for 5, 10 and 15 min.

2.2.2. Dehydration, infiltration and embedding in epoxy resin

Fixed samples were dehydrated in an ascending series of ethanol: 30, 50, 70 and 90 %, and three times 100% for 5 min each. In preparation of the resin infiltration, ethanol was replaced by propylene oxide (PO) in three steps (5/ 10/ 10 min). Agar low viscosity resin (LVR) (Agar Scientific, UK) was mixed under stirring: 12 g Agar resin, 2 g Hardener VH1, 11 g Hardener VH2 and 0.625 g accelerator. Resin infiltration was performed by raising the resin concentration in mixtures with PO: 1:3 for 20 min, 1:2 for 20 min, and 2:3 for at least 18 h overnight. A lid was placed on the vials to avoid evaporation of the solvent.

Freshly mixed resin was filled in PCR tubes used as embedding forms (Multiply®- Pro cup 0.2 ml, PP, Sarstedt). The transfer of the specimen into the Sarstedt tubes was done with feather tweezers under a binocular microscope placed in a vented hood. For infiltration with fresh resin the opened tubes were placed under vacuum (150 mbar) for at least 3 h. The polymerization was accomplished over two days in a polymerization oven at 60 °C

2.3. Cryopreparation of high- pressure frozen sample

2.3.1. Preparation

Three main samples were used: Hydrated cysts (12h), freshly released cysts harvested from the rearing bottles, and dissected ovisacs. Selected females were put on crashed ice to reduce their movements. Decapitation was done with a micro scissor, followed by removing the thorax and abdomen until the ovisac remained. Immediately before each shot of the high pressure freezer an animal was dissected and instantly frozen. For all samples 10 % Bovine serum albumin (BSA) in ddH_2O was used as a cryoprotective filler of the carriers.

For preparation of the carrier sandwiches, carriers of type A (6 mm diameter; 200µm depth) were combined with carrier type B (6mm diameter; flat surface) (Fig. 3).

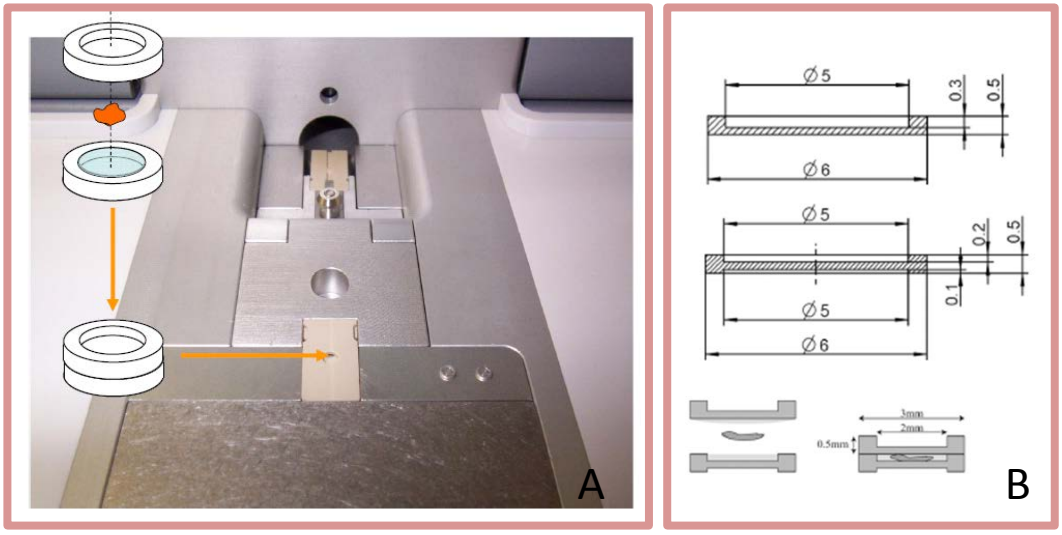

Fig. 3: Sample carrier arrangement in the high-pressure freezer HPM 100 (Leica). (A) Filled carrier sandwich, placed into the middle plate of a cartridge (B) Individual carriers type A and B (above), 6 mm in diameter, and combined as a sample-containing sandwich (below) (Kaech & Ziegler 2014)

Both carriers were coated with 1-hexadecene with a fine paintbrush, to facilitate the separation of the carrier sandwich and the liberation of the specimen from the carrier at the later stage of resin embedding.

The specimen was transferred into the 200 µm deep carrier (Type A) with the help of a preparation needle and feather tweezers 0.5 µl of 10% BSA was added with a Gilson pipette to

fill up the carrier completely. A carrier (Type B) was placed with its flat surface on top like a lid. Exertion of pressure on the top of the sandwich, with a preparation needle, resulted in the protrusion of excess BSA. Blotting of the extruding BSA with filter paper should ensure a proper sealing of the carrier.

2.3.2. Freezing

The filled carrier sandwich was inserted into the middle plate of the sample cartridge (Fig. 3A), resting on one of the two half cylinders and it was covered with the second half cylinder of the cartridge. HPF was initiated without delay at the touch of a button of the HPM100 (Leica). The cartridges with the frozen samples were released into liquid N_2. The sample carriers were stamped out of the middle plate and either stored in a liquid N_2 tank in cryo- tubes (cryo- vials, 2 ml; VWR) for later processing, or placed into Sarstedt tubes filled with frozen OsO_4/ acetone for subsequent FS.

2.3.3. Automatic freeze substitution under agitation

FS was performed in an automatic freeze substitution unit AFS2 (Leica) equipped with an agitation module with a capacity for eight Sarstedt tubes (2 ml).

A 10 ml solution of 1% OsO_4 in dried acetone was split into 8 tubes, which were immersed in LN_2. Carriers with HP frozen samples were placed onto the frozen substitution medium (1% OsO_4) in the tubes. Afterwards these were inserted into the AFS unit with its agitation module precooled to -140 °C. A motor with a magnetic rotor as driving force for agitation was set onto the lid of the AFS chamber and rotated with 20 Volt during the whole process.

Notably, the sample agitation was realized in absence of ethanol as a medium for cold conductance between the chamber wall and the sample tubes. The temperature sensor of the AFS is located underneath the chamber bottom, instead of being close to the sample. Therefore, a temperature gradient had to be considered for programming of the AFS. The actual

temperature curve was measured with a K- type thermocouple and USB data logger EL- USB-TC-LCD (Lascar electronics, US).

Based on this information, the temperature/ time schedule was programmed as follows: After a rapid warming up from -140°C to -105°C the programmed temperature was kept at -105°C for 10 h to ensure freeze substitution at -85°C. Warming up to -90°C in 2 h 30 min followed as next step.

After warming up from -90°C to -60°C within 2h, the temperature was kept constant for 2 h at -60°C. Subsequently the program temperature was raised from -60°C to +4°C (3h) and from +4°C to +20°C (1h). This was necessary for contrasting of lipid membrane with OsO_4.

The measured temperature/ time profile of this program, measured with the thermo- coupler placed inside a Sarstedt tube with acetone, is shown in Fig. 4.

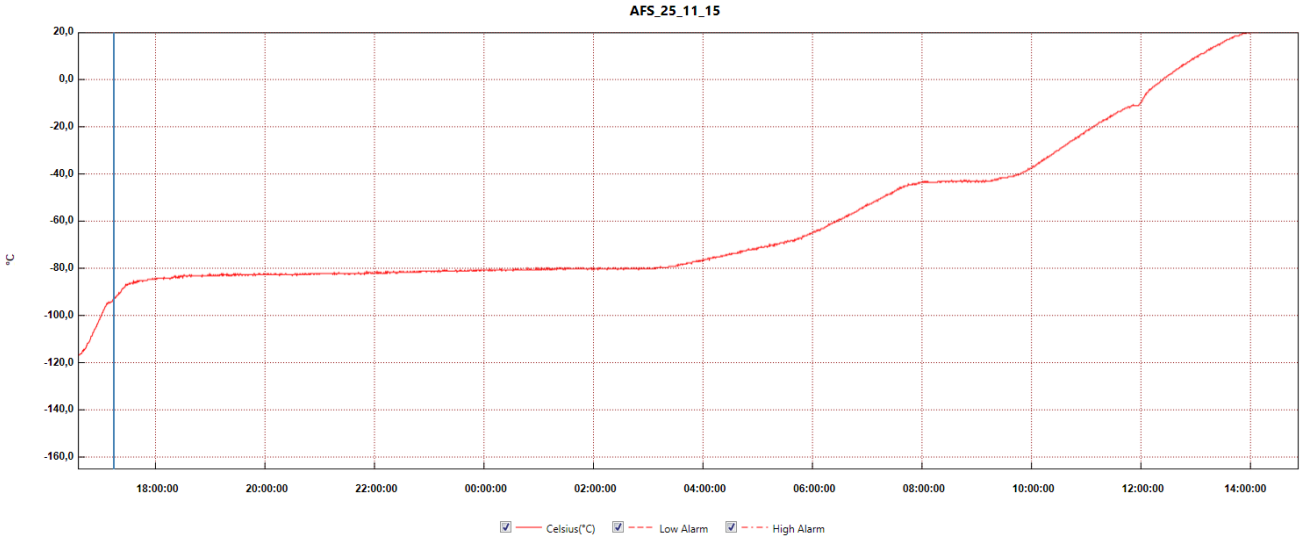

Fig. 4: Curve progression of the temperature of the AFS program described above, which was measured with a K- type thermocouple and USB data logger.

Subsequent washing and embedding of the samples took place outside the AFS. The OsO_4/acetone mixture was removed and samples, mostly inside their carriers, were washed three times with dried acetone (5/ 10/ 15 min).

2.3.4. Resin Infiltration and embedding

Fresh resin was mixed as described above. Subsequently the infiltration was performed by raising the resin concentration in mixtures with dried acetone stepwise: 1:3 for 20 min, 1:2 for 20 min, and 2:3 for at least 18 h overnight. A lid was placed on the tubes to avoid evaporation of the solvent. The following embedding and polymerization of the specimen was done as described above.

2.3.5. Sectioning and staining

Both, semithin and ultrathin sections were made on ultra-microtomes Ultracut S (Reichert) and UC7 (Leica). Semithin sections (2.0- 2.5 µm) were cut dry with glass knives, placed on glass slides and stained with 1% toluidine blue/ 1% Borax solution (5- 10 sec). Ultrathin sections (70 nm) were cut with a diamond knife (Ultra 45°; Diatome), placed on copper grids (200 mesh) coated with Formvar film (0.75 % Formvar in chloroform). Staining and contrasting was done with 0.5 % uranyl acetate (16 min) and subsequent 3% lead citrate (7 min).

2.4. Electron and light microscopy

Thin sections were observed with the TEM Zeiss Libra 120 at 120 kV. Pictures were made with the bottom mounted camera Olympus Sharp:eye TRS (4 Megapixel) and with the side port camera Olympus Morada G2 (11 Megapixel) and ITEM imaging software (Olympus softimage solutions GmbH).

Light microscopical pictures were made by Goldammer Helmuth and Sassmann Stefan in the Cell Imaging Lab at the University of Vienna, Althanstraße.

3. Results

3.1. Chemical fixation at room temperature

Initially, we tested the prospects for improvements of the chemical preservation of *Artemia franciscana* tissue by introduction of two measures, namely, MW-accelerated aldehyde fixation and the choice of PHEM-buffer made of Good´s buffer components that were adjusted to osmolality of the sea water (Montanaro et al., 2016). 4% PFA in PHEM buffer was applied, since PFA has the advantage to intrude into the tissue more rapidly (Hayat, 1990).

3.1.1. Tissue of nauplii of *Artemia franciscana*

The nauplii of *A. franciscana* have a size small enough to accomplish chemical fixation without the need of dissecting. In this case, the cuticle as a whole acts as a barrier against penetration of the fixative. For overall evaluation in the light microscope semithin sections of nauplii (ca. 2-4 µm in thickness) were cut and stained with toluidine blue. Fig. 5 displays a well-preserved nauplius that was cut transversely to get an overview of the animal's body. Head, thorax and abdomen are clearly distinguishable. Limbs for swimming are not shown, but parts of the intestine and the animal´s cuticle separating from the animals body due to chemical fixation, are visible.

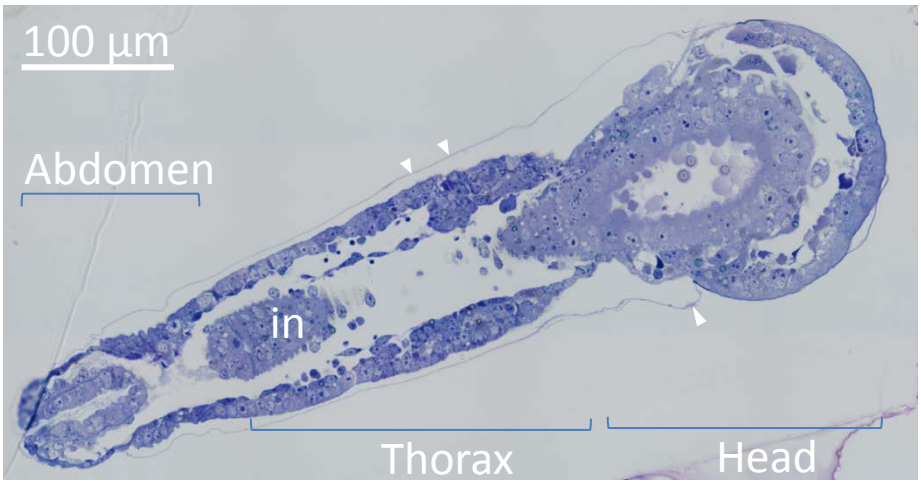

Fig. 5: Light microscopic overview of a transversal a section of a nauplius stained with toluidine blue. Head, thorax and abdomen are distinguishable. Also parts of the intestine (in) and the animal´s cuticle (arrowheads) are visible.

Ultrathin sections were used on the TEM has been used and to evaluate preservation of the same animal more distinctly at ultrastructural level: The tissues at the periphery and in the bulk of the sample were of varying preservation quality (Fig. 6 A-D). Some areas displayed a more electron-dense cytoplasm, which was rich in ribosomes and glycogen and mitochondria (Fig. 6A and B). Other regions of the sample were affected seriously by extraction of the biological material, resulting in an electron lucent cytoplasm with few ribosomes and rough endoplasmic reticulum (rER) with clearly widened lumen (Fig. 6B). Mitochondria and details like vesicles of unknown function (marked by an arrow) are distinguishable (Fig. 6B).

The cuticle itself shows extensions into the cytoplasm (Fig. 6C), which are possibly related to the formation of the new cuticle, while the old cuticle is about to be released during the molting process (Fig. 6C) (Criel & Walgraeve 1989). In the most areas of the tissue the cytoplasm was washed out almost completely and the remaining glycogen granules were aggregated (Fig. 6D).Despite of these variations in preservation quality, adequately preserved regions could be found, allowing hypotheses and conclusions about functions of several anatomical structures.

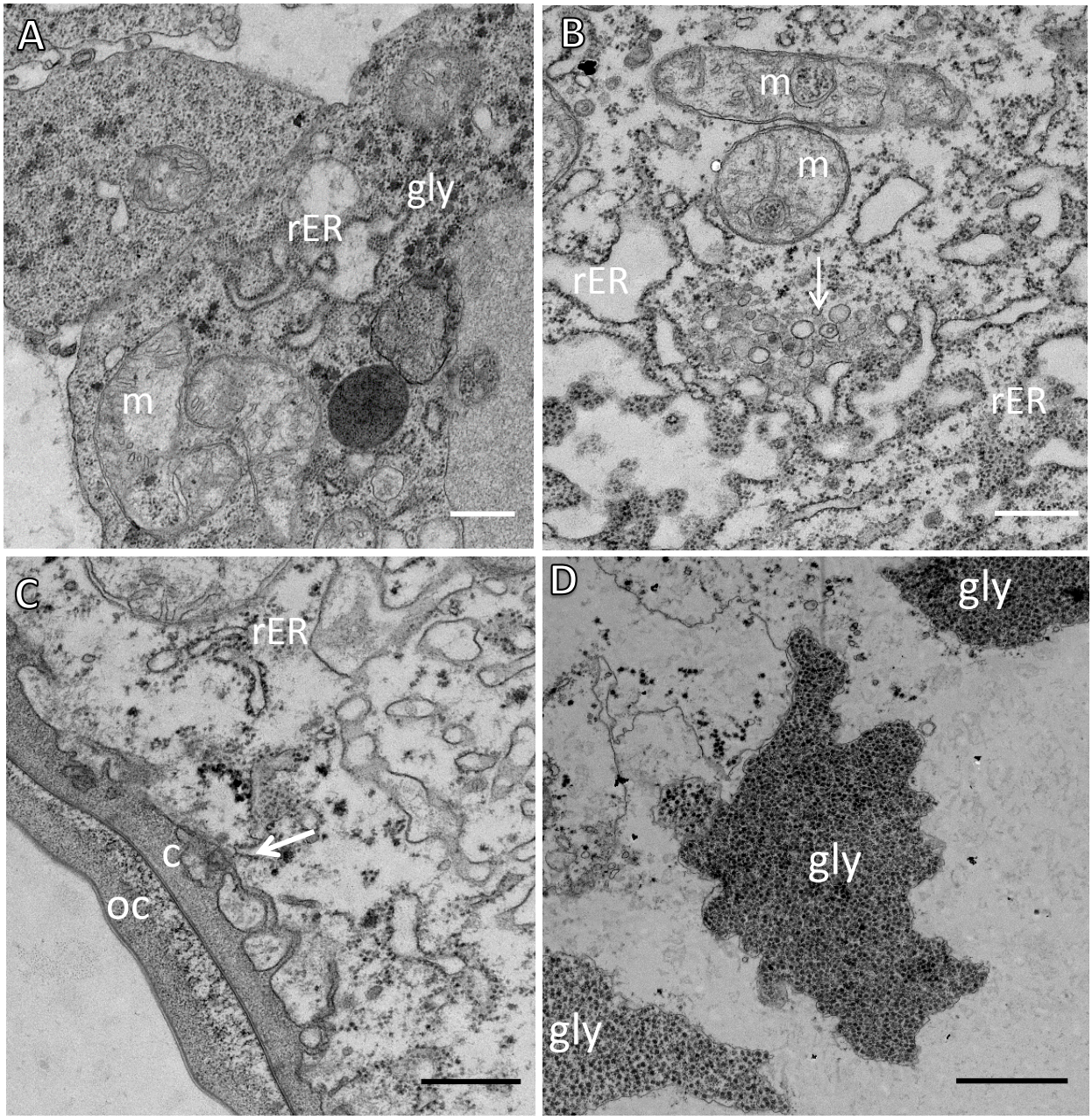

Fig. 6: TEM micrographs of different tissue areas of nauplii of *A. franciscana* chemically fixed at RT. A: At the periphery of the animal organelles like mitochondria (m) and rough endoplasmic reticulum (rER) are preserved. Glycogen (gly) accumulations are visible within thee cytoplasm that is more electron dense than in B and C. B: Details of mitochondria, rER and vesicles (arrow). C: Cells next to the cuticle (c) shows electron-lucent cytoplasm and swollen rER. oc, old cuticle; c, cuticle. D: In areas of massive extraction accumulations of glycogen (gly) are found. Bars: 500 nm.

An example for the potential of the conventional preparation at room temperature for studies of structural/functional relationships is our finding of muscle cells, arranged periodically along the basal laminar of the midgut epithelium (Fig. 7, arrows)(Bartolomaeus et al. 2009). According to Criel and Macrae (2002) these circular muscle ´belts´ are involved in peristaltic function of the intestine. The cross-sectioned filaments of actin and myosin, representing the contractile ´belt´, are resolved at higher magnification in the TEM (Fig. 7, insert).

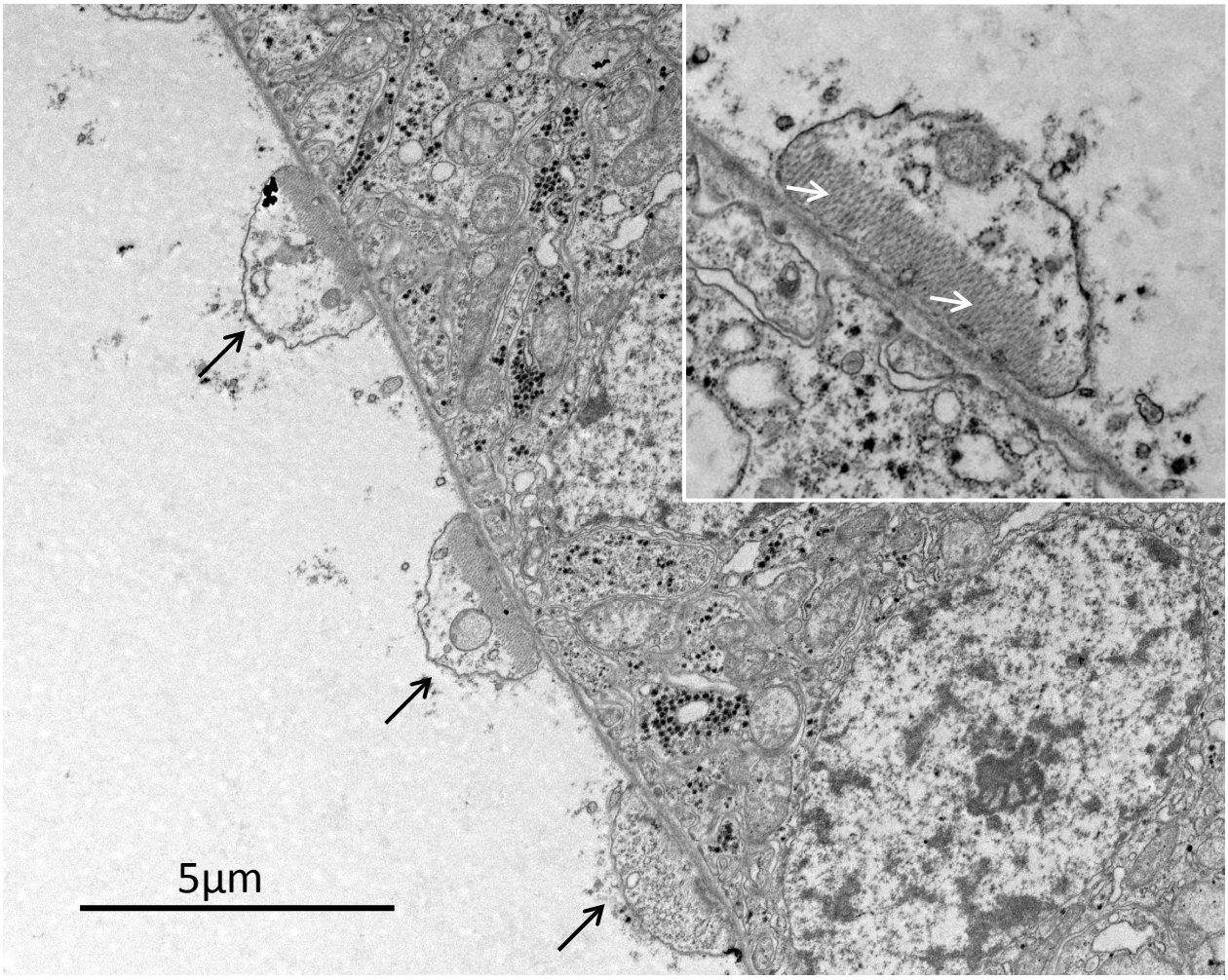

Fig. 7: TEM cross section of individual muscle cells (arrows), which are arranged as ´belts´ periodically along the basal laminar of the intestine. Filaments of actin and myosin are clearly visible at higher magnification (insert, white arrows)

3.1.2. Cysts with intact eggshells: A challenge for chemical fixation

Diapaused cysts were rehydrated for 6 hours and subsequently chemically fixed as described above. It turned out that chemical fixation, supported by MW exposure, could not achieve proper preservation of the eggs (Fig. 8). The inner and outer cuticle membranes, consisting of strongly cross-linked chitin molecules pose a barrier for both the fixative and the resin monomers (Sugumar & Munuswamy 2006). The resulting insufficient infiltration of the chemicals caused fixation- and embedding artifacts of the embryonic cells (Fig. 8). Thin sectioning of resin-embedded cysts endowed with intact eggshells was impossible, since incomplete infiltration with resin led to breaking out of the whole egg content. Nevertheless, a very small proportion of chemically fixed eggs could be sectioned for observation in the TEM. It turned out, for the non-perforated rehydrated cysts, that the embryonic cells were destroyed and the outermost layer, namely the alveolar layer of the eggshell, got lost during preparation (Fig. 8).

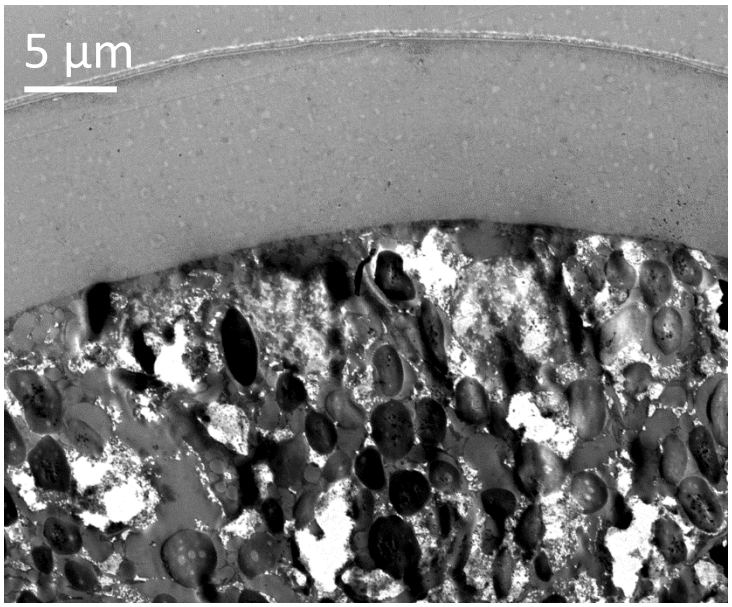

Fig. 8: Rehydrated cyst with intact eggshell chemically fixed with 4% PFA at RT. Intrusion of both the fixative and resin monomers was incomplete, resulting in bad preservation of the eggshell and embryonic cells.

3.1.3. Eggshells and the hatching nauplii

Much better prospects for preservation of embryonic tissue are given, when the eggshell is perforated. This happens naturally, during hatching of the nauplii (Clegg and Trotman, 2002). The burst of the eggshell, allows penetration of the fixative and the resin molecules. Consequently, the hatching process can be preserved in good quality for light microscopy. Fig. 9 displays a nauplius, while released from its eggshell. Cells are distinguishable and anatomical details can be identified. The eggshell itself is also well-preserved.

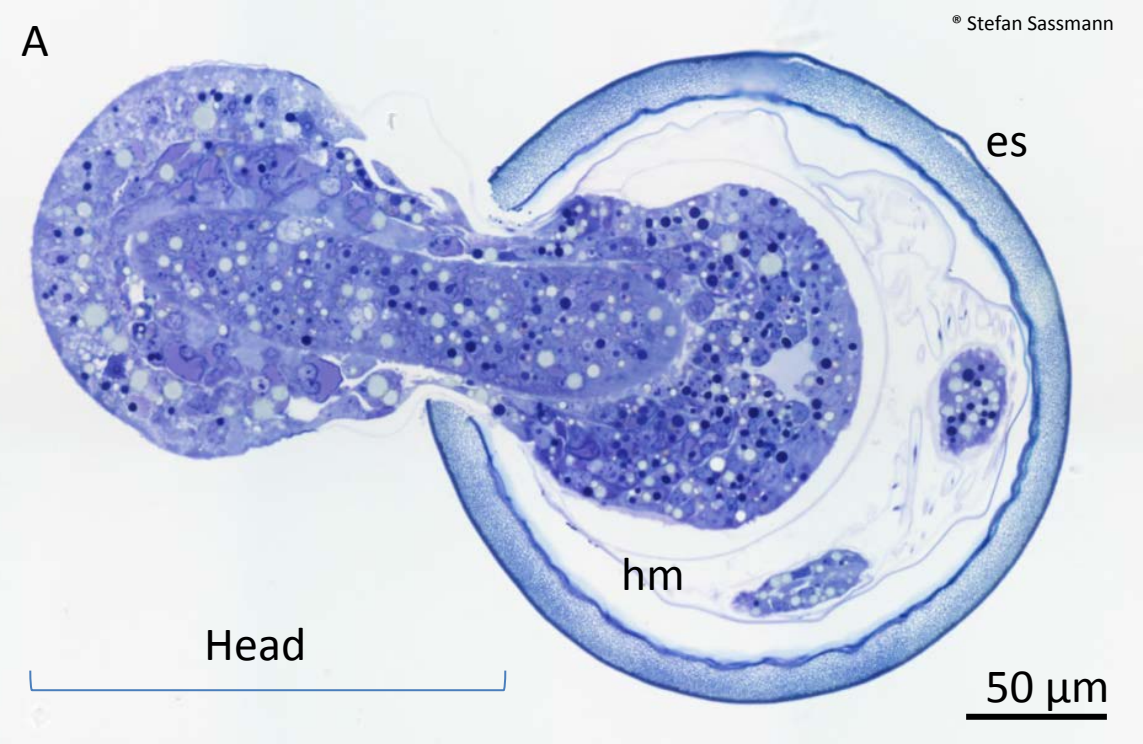

Fig. 9 A: Light microscopic view of a hatching embryo. The eggshell (es) is naturally ruptured by the hatching process. Embryonic tissue is well-preserved and individual cells can be recognized. hm, hatching membrane.

By using TEM, details of the hatching process, such as the eggshell fracture site and the so called hatching membrane became visible (Fig. 10). The hatching membrane covers the nauplius after egg rupture in the immobile “umbrella stage” until the animal frees itself by movements (Clegg and Trotman, 2002). The capacity of this membrane for unfolding is indicated by numerous folds visible only at higher resolution. Embryonic cells are well preserved and comparable with chemically fixed nauplii shown in Figs. 6 and 7. However, studies of the tissues of hatching

nauplii reveals also typical chemical artifacts that did not become apparent at light microscopical level. Fig. 11 displays a tissue area affected by extraction and cell shrinkage (see extracellular omissions marked by arrows in Fig. 11). Yolk platelets, glycogen accumulations, nuclei are recognizable despite of these serious tissue artifacts (Fig. 11).

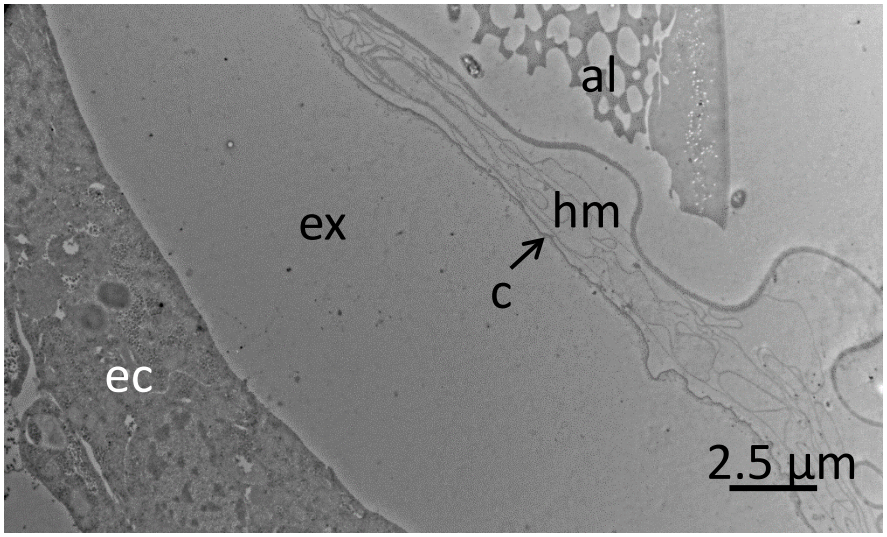

Fig. 10: TEM micrograph displaying a detail of the hatching process at the eggshell rupture site. Note the alveolar layer (al) of the eggshell and embryonic cells (ec) wrapped by the animal´s cuticle (c) and the hatching membrane (hm). ex, extra cellular space.

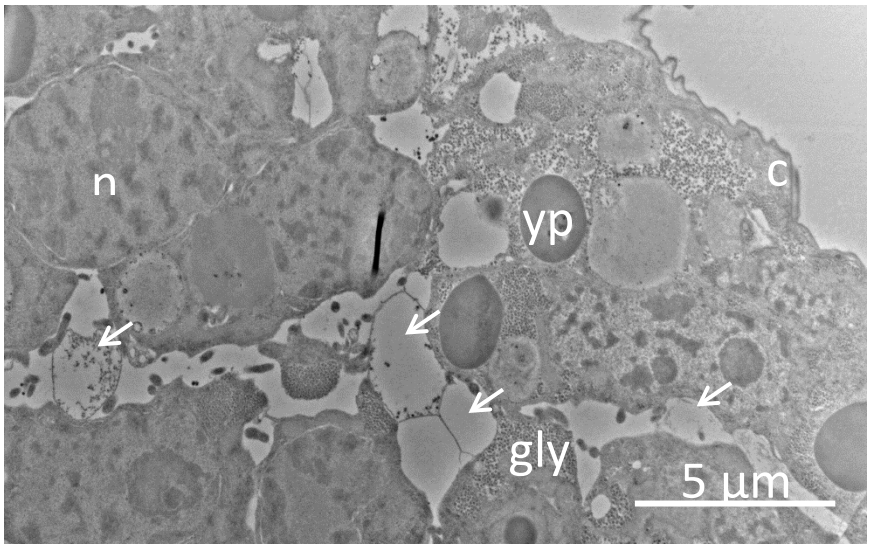

Fig. 11: TEM micrograph displaying tissue of a hatching nauplius chemically fixed at RT. Extraction of biological material results in omissions within the extracellular region (arrows). n, nucleus; , yolk platelet; gly, glycogen; c, cuticle.

3.2. Cryopreparation

In this study the prospects of cryopreserving tissues of *Artemia franciscana* were tested. For this we had to establish reliable handling procedures and protocols based on HPF and subsequent automatic FS. The following technical aspects had to be considered for the protocols outlined in the "Material and Methods" (page 11).

High pressure freezing

The animals were dissected using micro scissors and forceps already before getting in contact with the filler of the carrier, BSA. 20% BSA was initially favored as filler, because of its cryoprotective properties at high concentration. However, it turned out that the reduction of the BSA concentration to 10% BSA provided better results. The BSA concentration had also an impact on FS, including the subsequent handling of the sample. At concentrations high enough, in this case 10%, BSA crosslinks and forms a tablet around the sample, at first this seemed to be an advantage for further handling of the sample but it caused problems. Tablets formed by 20% BSA had the tendency to stick to the carriers and to break easily. This led frequently to the breakage or loss of the specimen. To reduce the stickiness, we applied just 10% BSA. Moreover, we coated both carrier parts with a thin film of 1-hexadecene by using a fine paint brush. These two adaptions of the method simplified the handling and the transfer of the freeze-substituted specimen after infiltration with LV-resin.

We tested sample carriers of different diameter for their suitability of freezing *Artemia franciscana* and decided to use carriers, 6 mm in diameter, instead of carriers, 3 mm in diameter. The dissected tissue and also the individual hydrated eggs could be inserted without any problem and quickly into the larger sample holders. A proper filling procedure has to ensure the sealing of the carrier sandwich prior to freezing. Overfilling of the 200 µm deep carrier (type A) with 10% BSA and subsequent closing of the sandwich with the plane side of the carrier type B acting as a lid, provided the possibility to squeeze the excess filler out of the carrier onto blotting paper. The filling, mounting and sealing of the carrier sandwich was done under an external binocular. Subsequently, the sandwich as a whole was transferred and inserted into the prepared middle plate of the cartridge, for subsequent freezing in the HPF machine. Although

the HPM100 itself is equipped with a binocular to support filling and mounting of sample carriers, we only got reliable results using an external binocular.

Automatic freeze substitution under agitation

Since there was an incentive to keep the FS protocol short, we started with a procedure under sample agitation for 3 hours at -100 °C, as successfully applied for other projects in the department. The distribution patterns of ice crystals indicated that such a protocol is too short for FS of shell-developing cysts in the ovisac and rehydrated cysts. At the same time, it became apparent that tissues which do not pose a strong hindrance for FS, such as cells of the cyst at a stage of vitellogenesis, can profit from short processing (not shown).

To achieve more ubiquitous applications to *A. franciscana* tissues we had to extend the processing time. Nine hours at -100 °C brought well preserved cells, as long as they did not contain large amounts of glycogen. If organized in large clusters, glycogen was regularly interspersed with ice crystals, because of its high affinity to water. Consequently, we used the presence of glycogen as indicator for fine-tuning of the time schedule. Extension of the substitution period to ten hours followed, which brought well preserved tissues in absence of glycogen-related crystallization artifacts.

(The following chapters should give an overview and also a comparison, especially in relation to the artifact like extractions, to chemical fixation at room temperature like described above.)

3.2.1. Tissue of *A. franciscana* nauplii

Nauplii, the larvae of *Artemia* sp., have a cuticle of ca. 100 nm minimum thickness (Fig. 12), which is formed by a layer of epithelial cells. The cuticle of nauplii is thin, compared with that of the adults, and is normally not a hindrance for FS under agitation overnight. Consequently, the epithelial cells underneath the cuticle are well preserved. They contain rER and mitochondria with a matrix that appears slightly more electron-dense than the surrounding cytoplasm (Fig. 12). The cisternae of the Golgi apparatus are well preserved (Fig.13). Glycogen granules, organized as rosettes and in absence of ice crystal damage, are dispersed throughout the cytoplasm (insert Fig. 13).

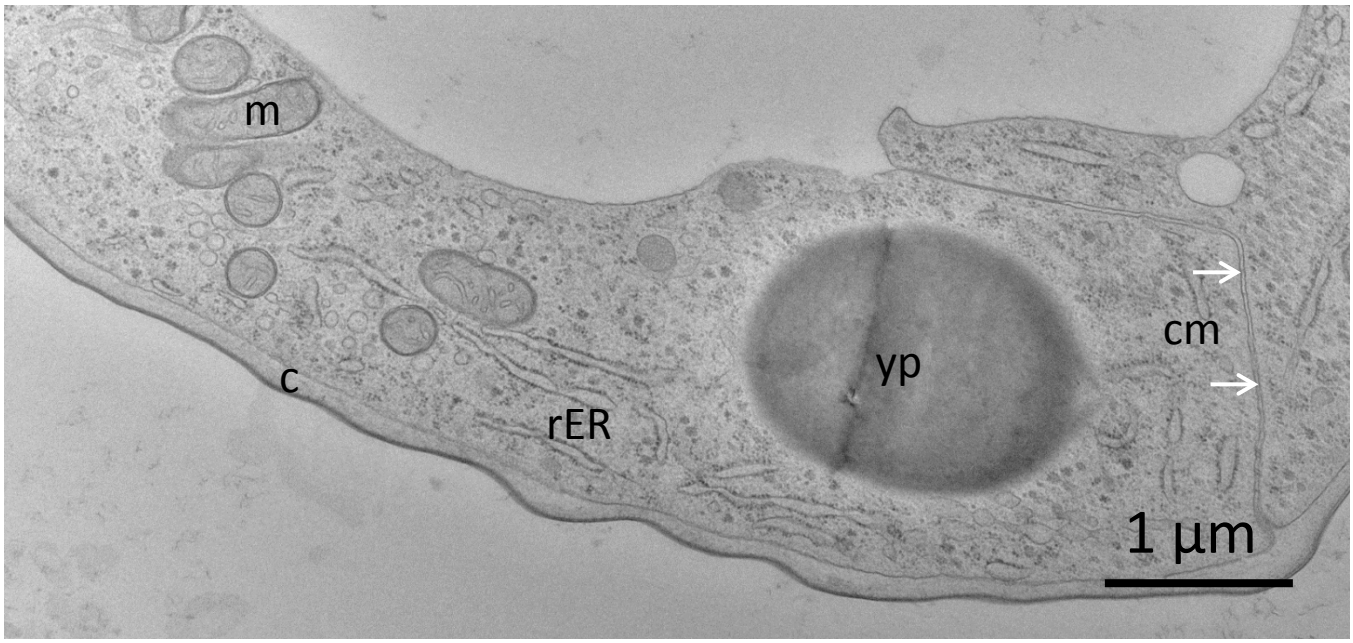

Fig. 12: TEM micrograph of a cryopreserved layer of epithelial cells covered by a thin cuticle layer. C, cuticle layer; rER, rough endoplasmic reticulum; cm, cell membrane; m, mitochondrion; yp, yolk platelet.

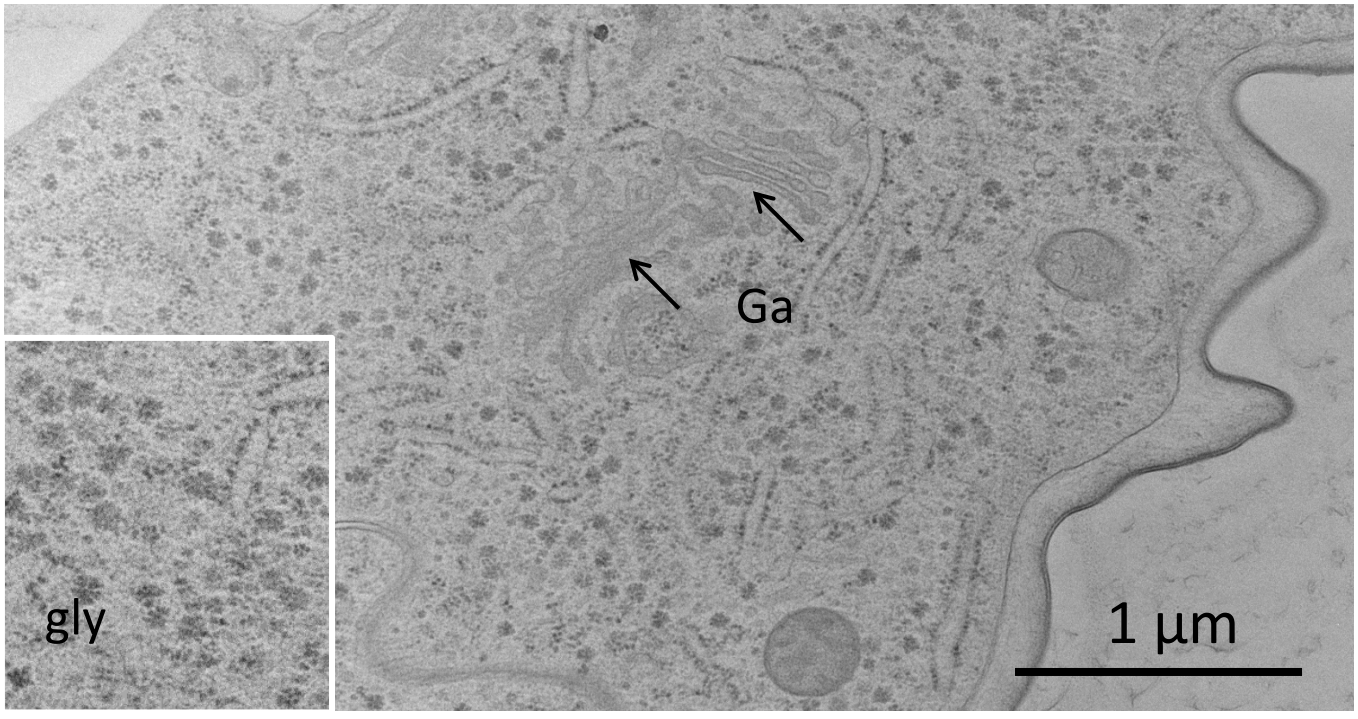

Fig. 13: Cell of epithelial layer with organelles like Golgi apparatus (Ga), rER, mitochondria and glycogen (gly) granules organized in rosettes (insert), which are well preserved by cryopreparation.

Fig. 14 displays cells of the somatic cells of the thorax indicating adequate freezing into the depth of the samples as expected by application of the HPF technique. The cell contours are lined smoothly by cell membranes, which are locally in contact with neighboring cells (white arrows). Nuclei, otherwise known to be prone to chromatin segregation by ice crystals, do not show such freezing artifacts. Strikingly, the cryopreparation preserves glycogen in form of large clusters, resulting in an electron-dense overall appearance of the glycogen domains within the cytoplasm (Fig. 14). Other areas of the cytoplasm contain numerous mitochondria, rER and autolytic vacuoles with heterogeneous content.

At higher magnification the quality of the preservation of the mitochondria becomes apparent (Fig. 15). They display smooth double membranes and cristae that were cryoimmobilized instantly in their positions prior to sample processing. Ribosomes exist both in their membrane-bound form, as rER and the outer nuclear membrane, and dispersed within the cytoplasm (Fig. 15).

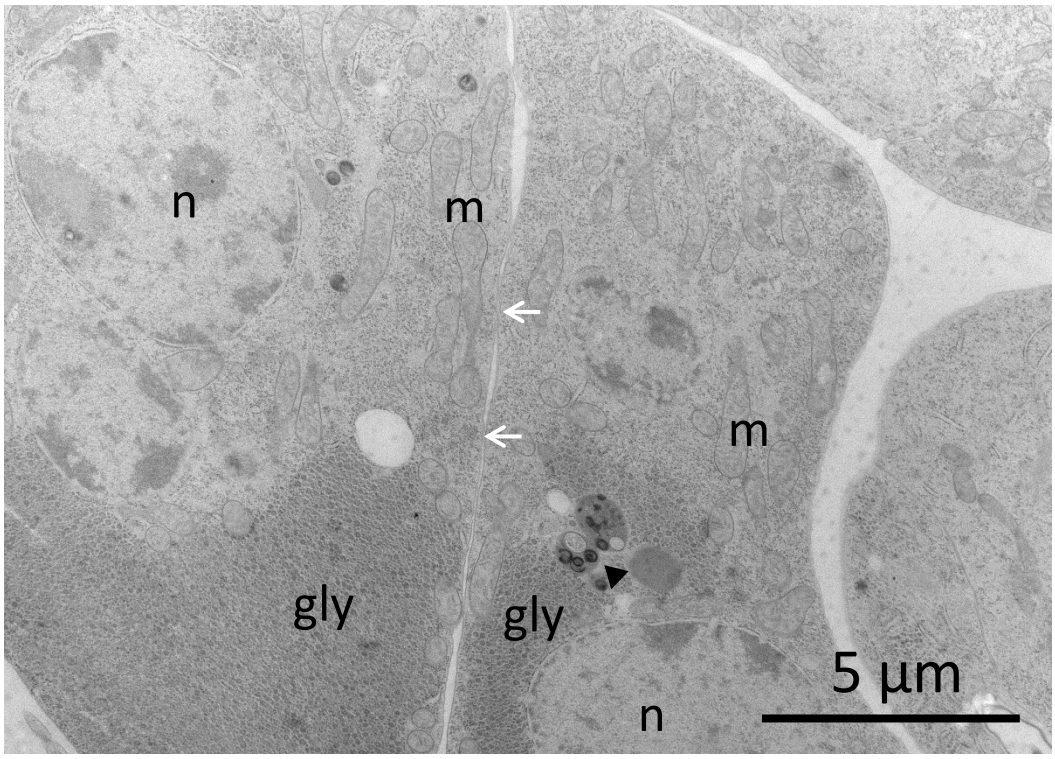

Fig. 14: TEM micrograph of cryopreserved cells of somatic cells of the thorax of *A. franciscana*. n, nucleus; m, mitochondrion; gly, glycogen clusters; cell membrane contact of neighboring cells (white arrows).

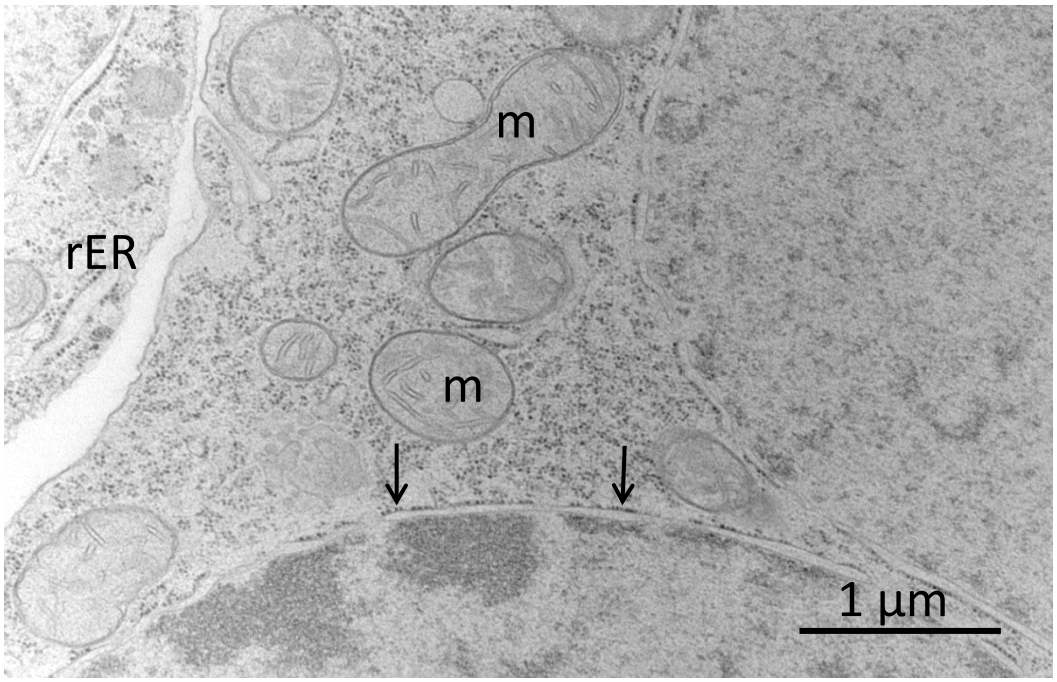

Fig. 15: TEM micrograph of cryoprocessed cells, showing the quality of cryopreservation of the inner tissue in detail. Note smooth mitochondrial and nuclear membranes. The latter are decorated with ribosomes (arrows). m, mitochondrion; rER, rough endoplasmic reticulum.

3.2.2. The dissected and cryopreserved ovisac of the adult females

The female reproductive system of *A. franciscana* has paired lateral ovaries, in which oocytes develop. By passing the adjacent oviducts into a single median ovisac (Fig.16) oocytes can be fertilized. The ovisac contains shell glands, which are responsible for the formation of one particular layer of the cysts eggshell, namely the alveolar layer, during oviparous reproduction. The newly fertilized eggs are endowed only with a thin transparent shell. At a later stage of development the pigmented alveolar layer covers the cysts, resulting in the brownish dark color.

Fig. 16: Stereo light microscopy of a dissected ovisac of *A. franciscana* showing the median ovisac with pale eggs inside.

HPF and cryoprocessing by FS opened new possibilities for the preservation and structural investigation of the whole female reproductive system. Developing eeggs and the formation of their shell within the ovisac can now be documented at high quality. It turned out that the incompleteness of the shell development for eggs within the ovisac was a technical advantage

for the FS process. Completed eggshells of cysts, in comparison, posed a serious barrier for FS (see next section).

The unfertilized oocytes are in the state of vitellogenesis in absence of any shell layer. In addition, the ovisac contains shell- producing gland cells, beside nurse and somatic cells, which appear dark blue after toludine blue staining of the semi-thin section (Fig 17). The cuticle and its adjacent epithelial layer are also clearly visible.

Important for the mounting of these samples for freezing with the HPM100, the ovisacs and their content were flexible enough to be fitted into 200 µm deep sample carriers. Under these favorable geometric conditions, optimal freezing throughout the samples became possible.

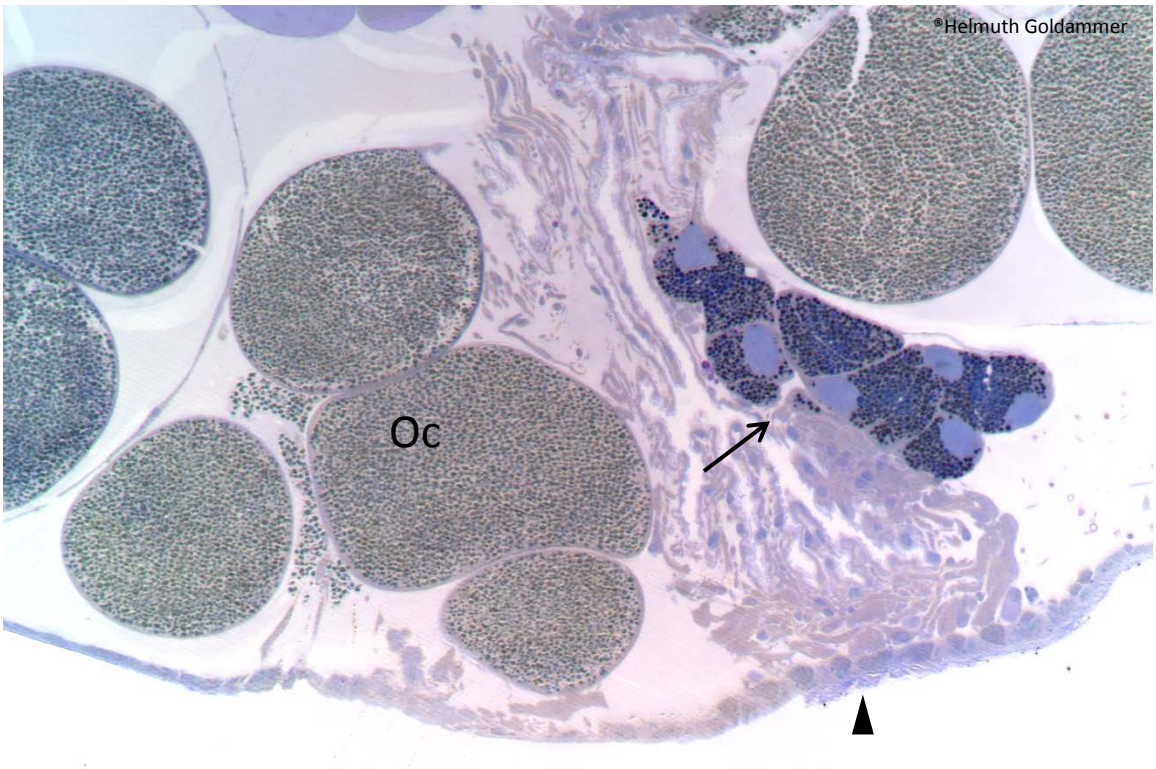

Fig. 17: Light microscopic section of the median ovisac containing developing oocytes stained with toluidine blue. Arrow, shell gland cells; Oc (oocyte); arrowhead, epithelial layer with cuticle.

3.2.3. The cuticle of the ovisac

The ovisac´s cuticle, which is shed periodically in the course of the molting cycle, consists mainly of chitin. Underneath this relatively thick cuticle layer is an epithelial layer, which produces the cuticle (Criel & Walgraeve 1989). As seen in Fig. 18 the cuticle is intimately connected with the epithelial cell that contains a nucleus. The elongated nucleus contains numerous patches of electron dense heterochromatin. Typical for cells of this epidermal layer the cytoplasm contains glycogen at a very high density. In spite of this it does not show any affection by ice crystals as it would happen if the substitution during the FS was not completely finished. Towards the inside, the epithelial cell is associated with other somatic cells of the maternal tissue, including those of muscle and cells rich in rER.

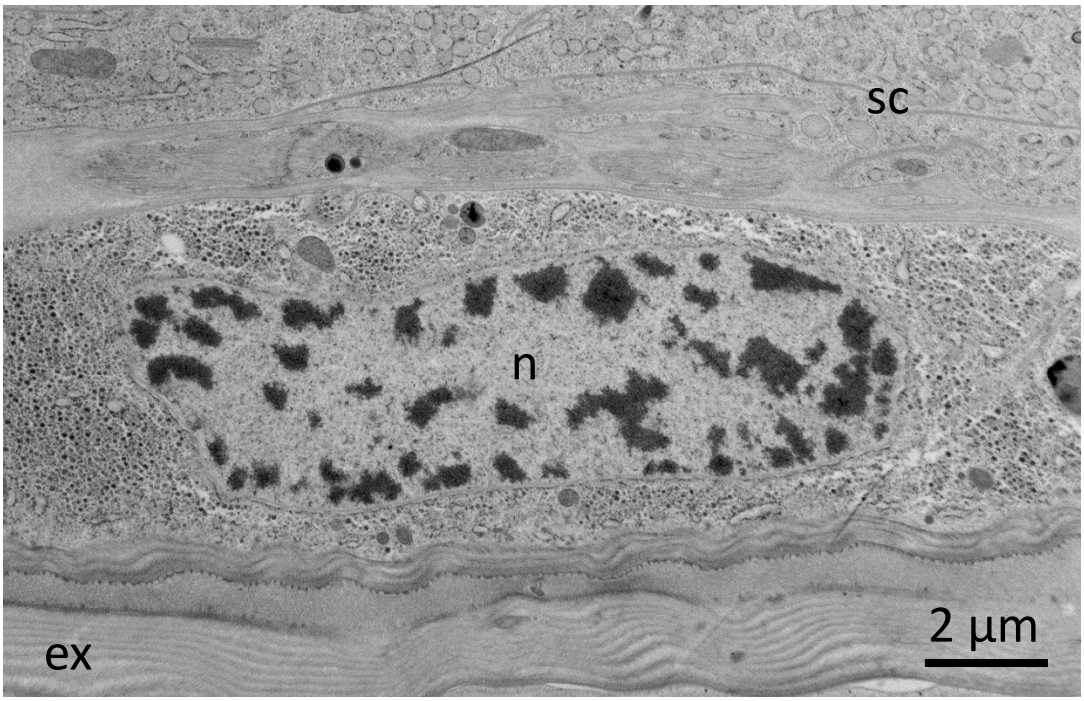

Fig. 18: TEM micrograph of a cryopreserved epithelial cell layer with adjacent new cuticle, which is about to be formed and old cuticle to be shed. In the old cuticle the exocuticle (ex) can be clearly distinguished. n, nucleus; sc, somatic cells rich in rER.

A layered cuticle associated with the epithelial cell of the epidermis is displayed in Fig. 19 in more detail. The different layers of the cuticle, namely epicuticle, exocuticle and endocuticle are clearly distinguishable (Criel et al., 1989). Additionally, another layer of brush-like structures at the outermost side of the epicuticle became visible in our cryo preparation (Fig 19), which would probably collapse in conventional chemical fixation. Between the layered cuticle described above and the epidermal cell, Fig. 19 displays additional cuticle-like layers perhaps representing a state of the formation of new cuticle, which has to occur in preparation of the molting process. The supposed "new" epicuticle is endowed with structures which resemble in part the brush-like structures of the outermost layer of the "old" cuticle, although they are smaller. Whether these structures are functional as a part of the molting process waits to be elucidated in the future.

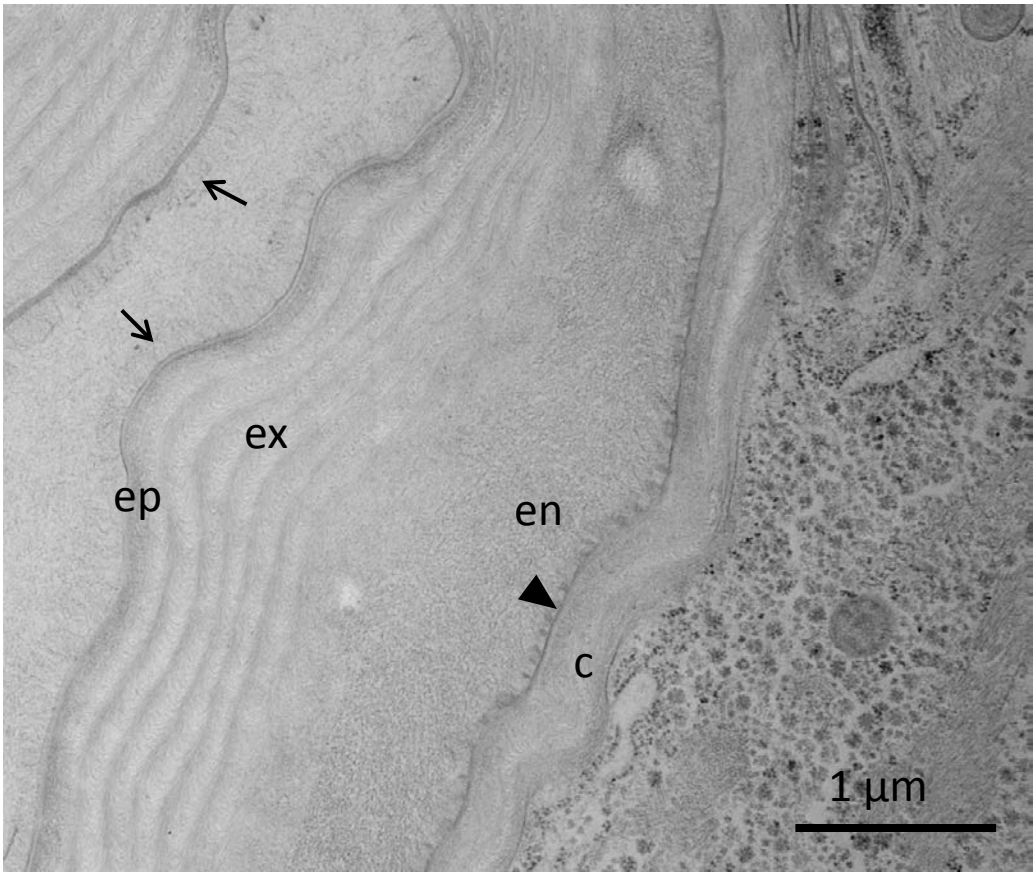

Fig. 19: Detail of the cryopreserved multilayered cuticle of the median ovisac of *Artemia franciscana*. Brush-like structures on the outermost side of the cuticle, which layers are clearly distinguishable in epicuticle (ep), exocuticle (ex); endocuticle (en), appear (arrows). One cuticle layer supposed to represent cuticle (c) that is forming anew during the molting process (arrowhead).

3.2.4. Cellular Inclusions in the epidermis: A novel observation

Cryoprocessing of the ovisac as a whole led to the surprising discovery of electron dense rhomboid- shaped inclusions in the epidermis which are aligned to fascinating ´zebra stripe´-like patterns as shown in the overview picture (Fig. 20, arrows). Notably this finding was restricted to epithelial cells of the ovisac lining cell type.

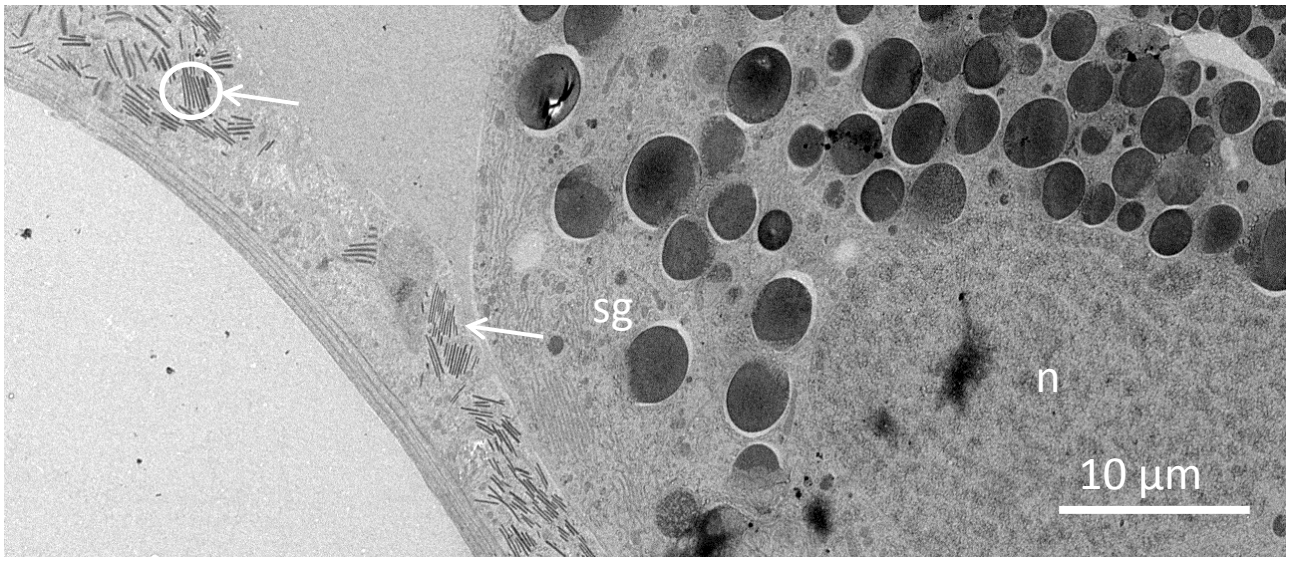

Fig. 20: Part of the cryopreserved median ovisac showing the cuticle with adjacent epithelial cells containing accumulations of black, rod- like inclusions (arrows). Note also the shell gland cell (sg) with a prominent active nucleus (n).

A closer look at higher magnification reveals that the white gaps between the black rod-like structures are devoid of material typical of the cytoplasm, such as ribosomes or glycogen (Fig. 21). The alternating black-and white patterns are organized in crystal-like ´flakes´ ranging from 1 to 10 µm in size. Depending on the section plane they appear either broader with washy interfaces, or straight with precise edges. They seem also to be flexible, since some inclusions are curve and they don't seem to have a preferential orientation. A roundish densely packed accumulation of very small rhomboid inclusions may be an important evidence for the formation process of these structures. Interestingly, the occurrence of ´flake´-like cellular inclusions is not limited to the cytoplasm. Rarely, we observed them also inside of the epithelial cell nuclei (Fig. 23).

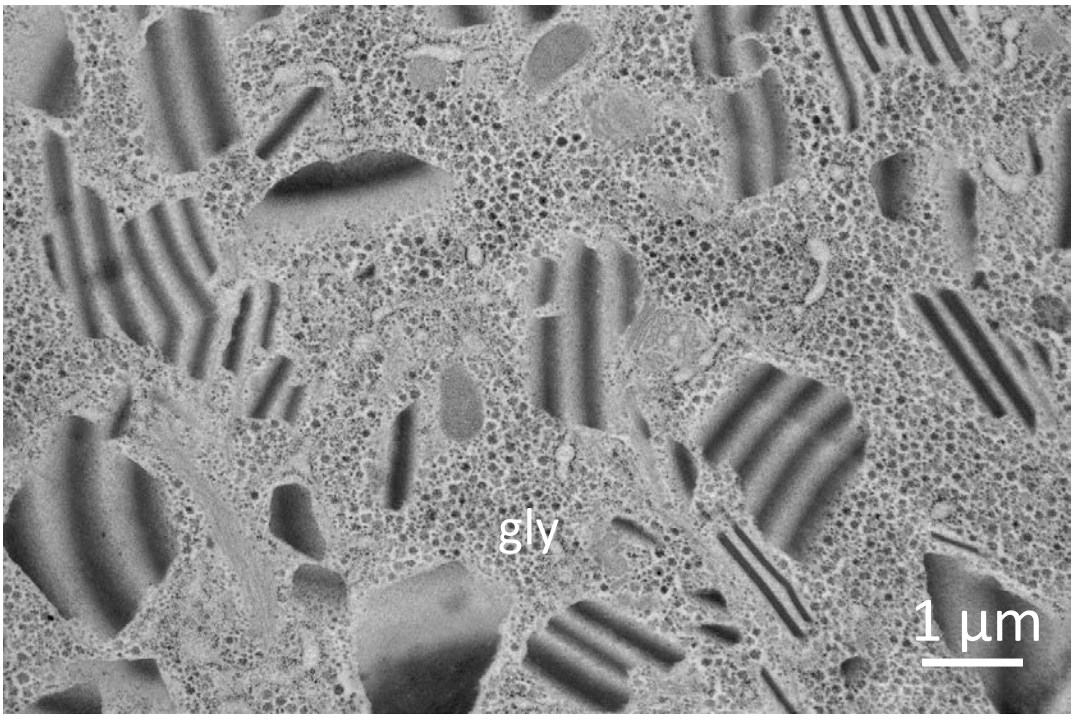

Fig. 21: ´Flake´- like inclusions in the cytoplasm of a cryopreserved epithelial cell at higher magnification. Note sharp edges at the outside of the ´flakes´ and the absence of ribosomes and glycogen in between the parallel running rhomboids that are electron dense. The inclusions are surrounded by glycogen accumulations (gly).

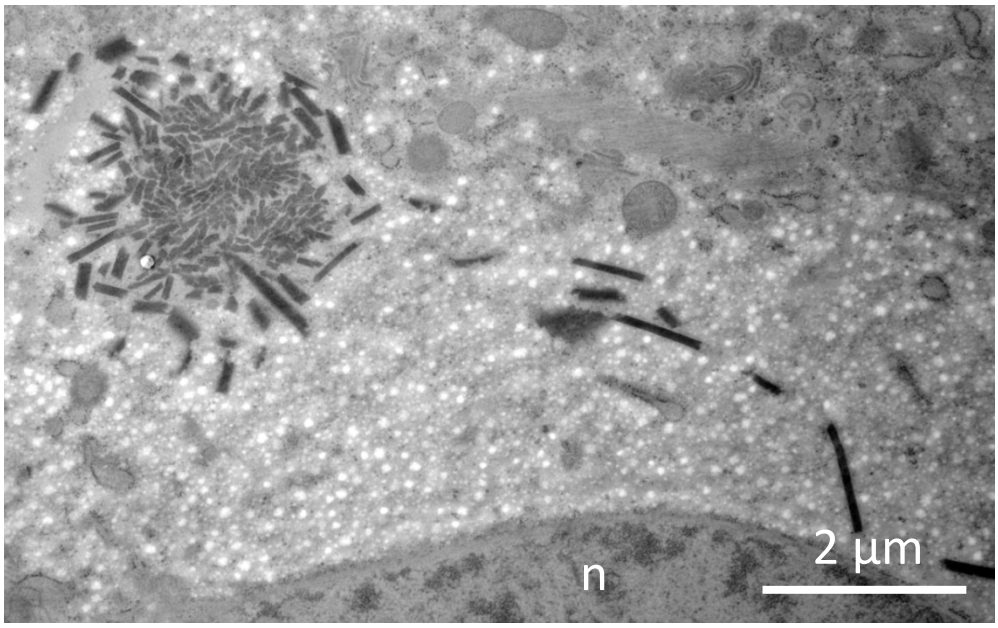

Fig. 22: Accumulation of the rhomboid inclusions in a roundish pattern. Smaller inclusions are accumulated at high density in the center. Given the extreme density of the cellular inclusion, we expect to get a hint, how these structures are initiated and formed, if a sufficient amount of sections is evaluated in the TEM. Note also small ice crystals artifacts in the cytoplasm but not in the nucleus (n).

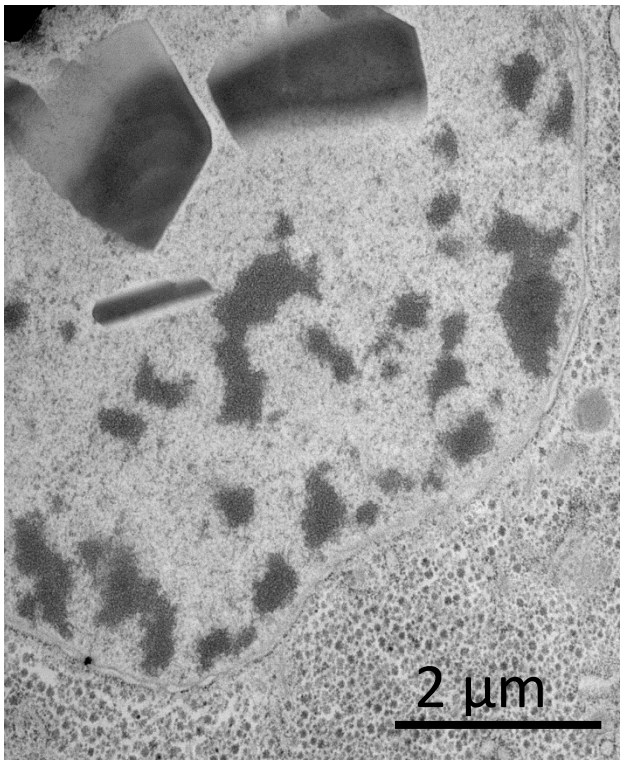

Fig. 23: ´Flake´- like inclusions in the nucleus.

The discovery of these crystal-like inclusions in the epithelial lining of the ovisac initiated the starting point for a number of ongoing cooperation that all aim at the elucidation of the nature and possible purpose of these extraordinary structures (see Discussion).

3.2.5. Study of the egg development in the ovisac based on cryopreservation

Cryopreservation of the ovisac for the first time provided insights into egg development on basis of instant cryoimmobilization. In the following, data are presented in evidence for the potential of this approach for future systematic studies of the maturation of oocytes during oogenesis, inclusively of the yolk platelet formation during vitellogenesis.

3.2.5.1. The ovary: maturing oocytes during vitellogenesis

Vitellogenesis, the process of yolk synthesis and storage, occurs before fertilization. Figs. 24 and 25 show oocytes with a huge amount of dark proteinaceous yolk platelets and smaller yolk droplets which are brighter in contrast (Criel, 1992). The latter are supposed to be of lipoid nature, although this is not supported by the degree of osmification of these structures during FS (Hayat 1990). That may be because of different molecular composition of these lipids.

At higher magnification crystalline needle-like inclusion within the yolk platelets become visible, which tend to aggregate in foci (Fig. 25, arrows). Numerous glycogen granules, organized in rosettes, were preserved in the vicinity of the yolk platelets in absence of ice crystal artifacts.

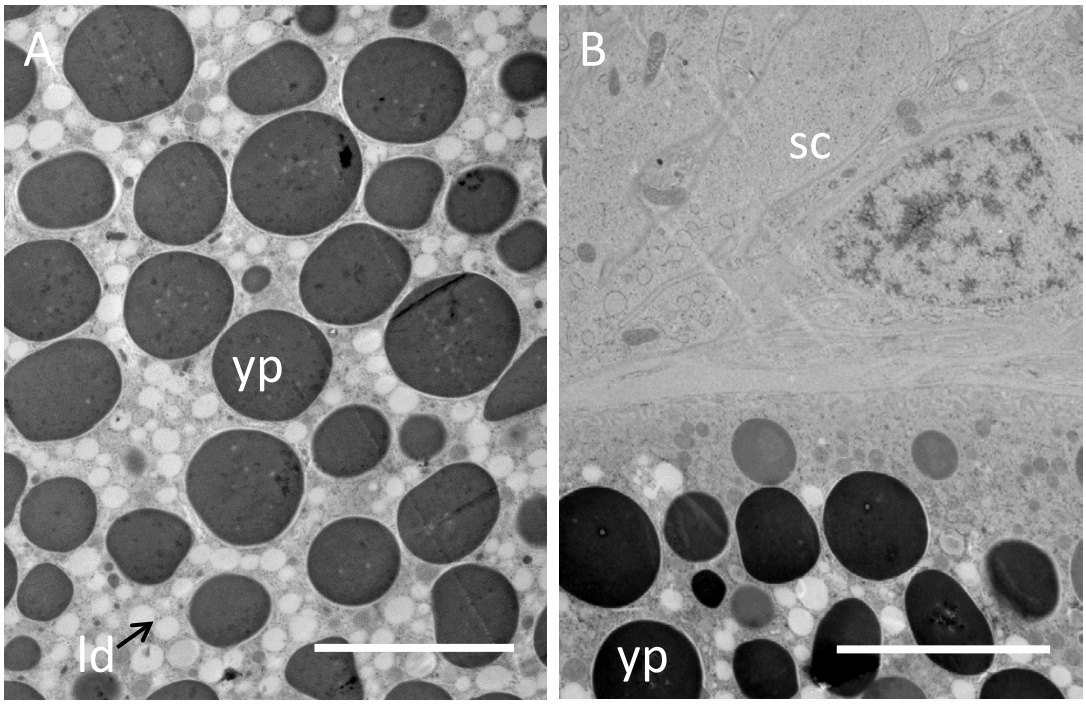

Fig. 24: Electron micrographs of maturing oocytes during vitellogenesis. A: Accumulation of proteinaceous yolk platelets, yp; and lipid yolk droplets, ld (arrow). B: Oocyte surrounded by somatic cells (sc); Bars: 5 µm.

As described previously by Criel (1992), sections of oocyte contain occasionally so-called ´nuclei´ as centers of metabolic activity of yet unknown function (Fig. 26). These ´nuclei´ accumulate many small vesicles. The existence of such vesicular aggregates was confirmed in our cryopreserved samples (Fig. 25). As indicated at higher magnification, the cryotechniques applied, led to the preservation of the internal details of these vesicles, which would be lost by chemical fixation and processing at RT (Criel, 1992). While some of these vesicles are homogeneous others contain heterogeneous content of yet unknown origin (Fig 25 B). Those homogeneous vesicles, which are dark in contrast, might be considered as precursors for much larger YP.

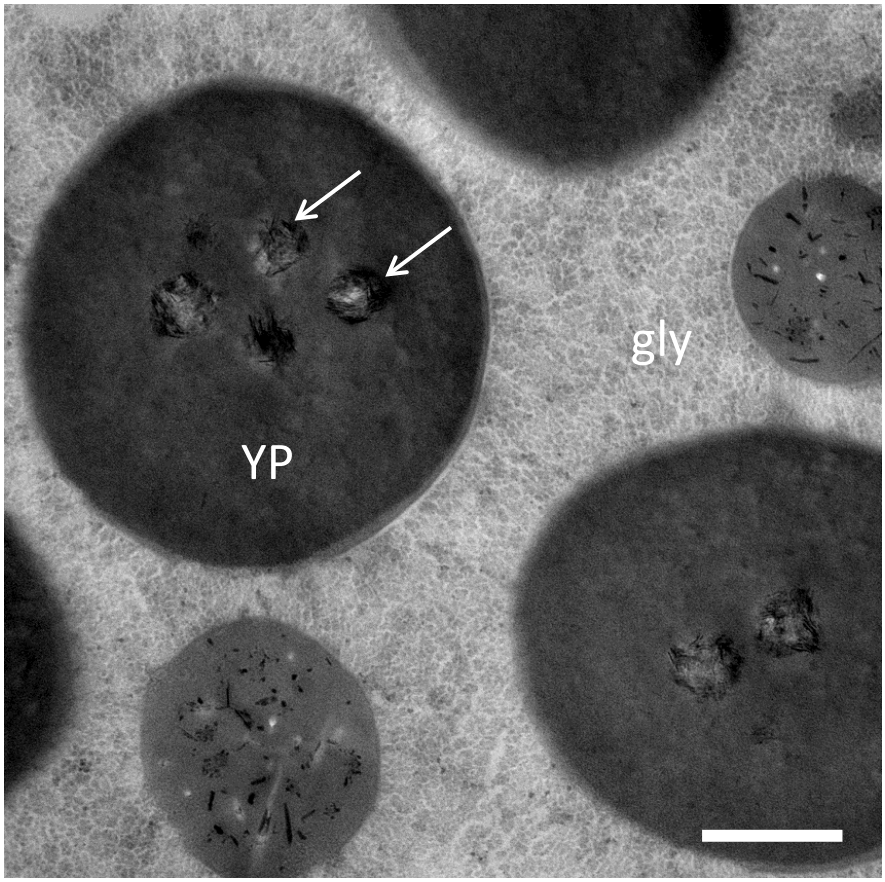

Fig. 25: High magnification of yolk platelets with crystalline inclusions surrounded by glycogen rosettes. Crystalline inclusions of yolk platelets are shown (arrows); YP, yolk platelets; Gly, glycogen. Bar: 500 nm.

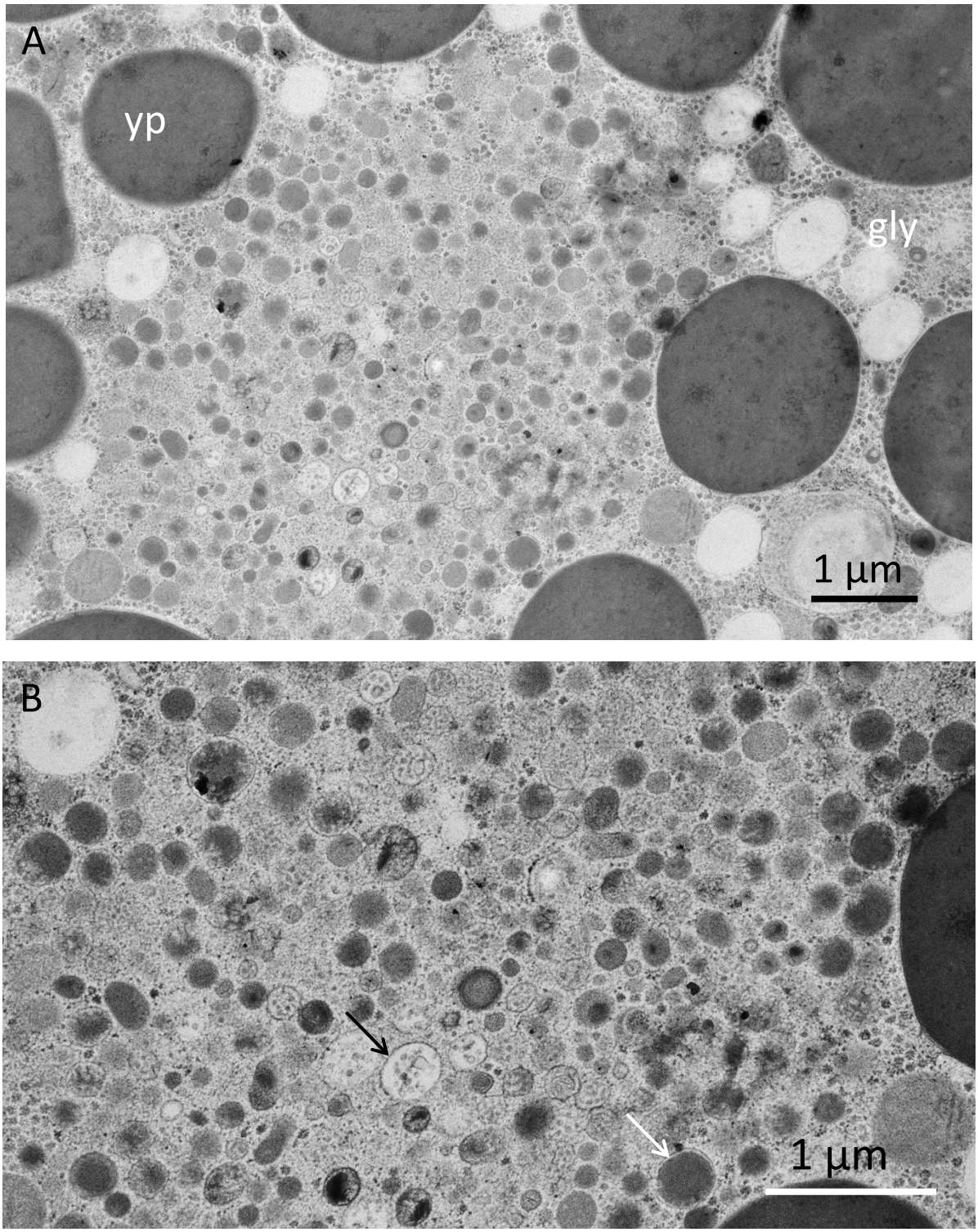

Fig. 26: Yolk “nucleus” located inside of a cryopreserved oocyte during vitellogenesis. A: Cluster of vesicles surrounded by yolk platelets (yp) and glycogen (gly). B: Detail displaying vesicles with heterogeneous (black arrow) and homogeneous (white arrow) content. Some of the latter might be precursors for the formation of large yolk platelets.

3.2.6. Developmental stages of the cyst in the ovisac

If the environmental circumstances are not favorable, developmental steps lead to the formation of eggs, which later on are arrested in diapause. We observed that the eggshell formation takes places in sequential steps: At first the outermost alveolar layer is formed maternally by shell glands inside the ovisac. Afterwards the inner and outer cuticle and the fibrous layer in between, all from embryonic origin, emerge. Even though the precise steps of development are not clearly described yet, the following results give interesting insights of developmental stages of cysts.

Cysts, when they are released from the mother's ovisac, stop their development in the early gastrula state. Preparing diapaused eggs, still developing in the ovisac, brought insight into the previous stages of development. In our findings, not only the eggshell but also the cell formation was still in progress.

In Fig. 27 the outermost embryonic cells are shown, lying next to the eggshell, containing nuclei and mitochondria, having the capacity for further division. The rER is mainly represented around the nucleus and accumulations of glycogen are evidence for active processes in these cells (Fig. 27). Once the alveolar layer is formed the embryonic cuticle begins to arise as shown in Fig. 27, which displays an early state of the embryonic cuticle formation. Using cryopreparation it became possible to visualize extracellular cell components and vesicles, which could possibly indicate important details related to the development of the eggshell or other transport mechanisms. Besides these cells, the embryo contains cells without nuclei, which are filled with nutritious content, organized mainly as yolk platelets and glycogen (Fig. 28). These cells support energetically to develop to a hatching embryo after diapause.

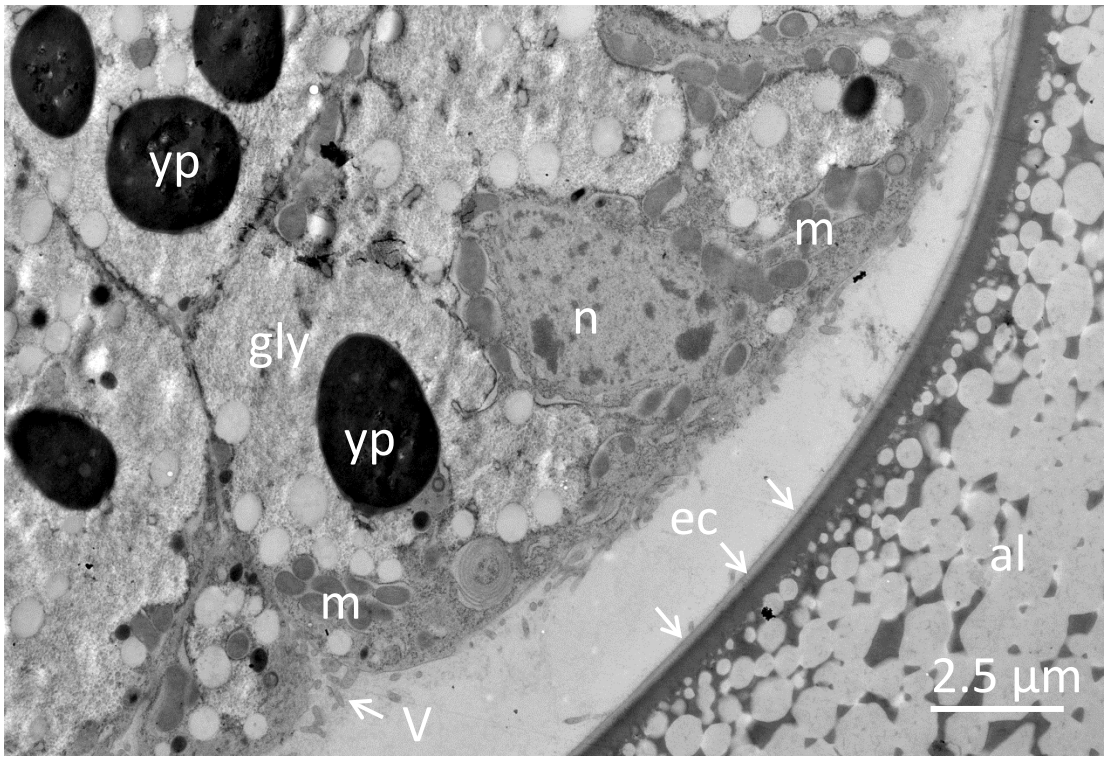

Fig. 27. Overview electron micrograph of a developing cyst inside the ovisac: Cells of the embryonic tissue are separated from the eggshell represented by an embryonic cuticle (ec) probably formed by help of vesicles (v) and the alveolar layer (al). Cells include yolk platelets (YP), Glycogen (gly), lipid yolk platelets (ly), mitochondria (m) and nucleus (n).

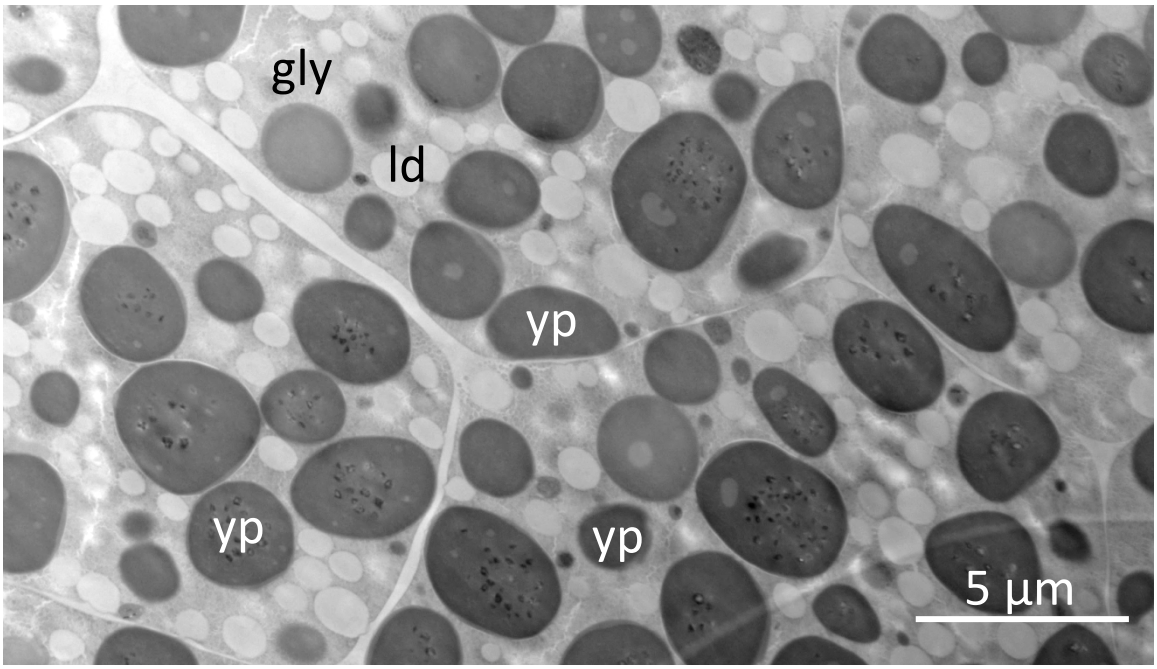

Fig. 28: Inside the developing embryonic tissue cells with mainly yolk platelets (yp), lipid yolk droplets (ld) and Glycogen (gly) is found.

3.2.6.1. "OSERs" as specific details of developing cells of cysts in utero

The following electron micrographs provide interesting insights of interacting cell components of the developing cyst. The preparation quality of different organelles and non-membrane bound constituents are evidence for advantages by using HPF and AFS. In addition to the cells themselves, the extracellular space is well-preserved and contains information, which might be relevant for studies of intercellular transport (Fig. 29, insert).

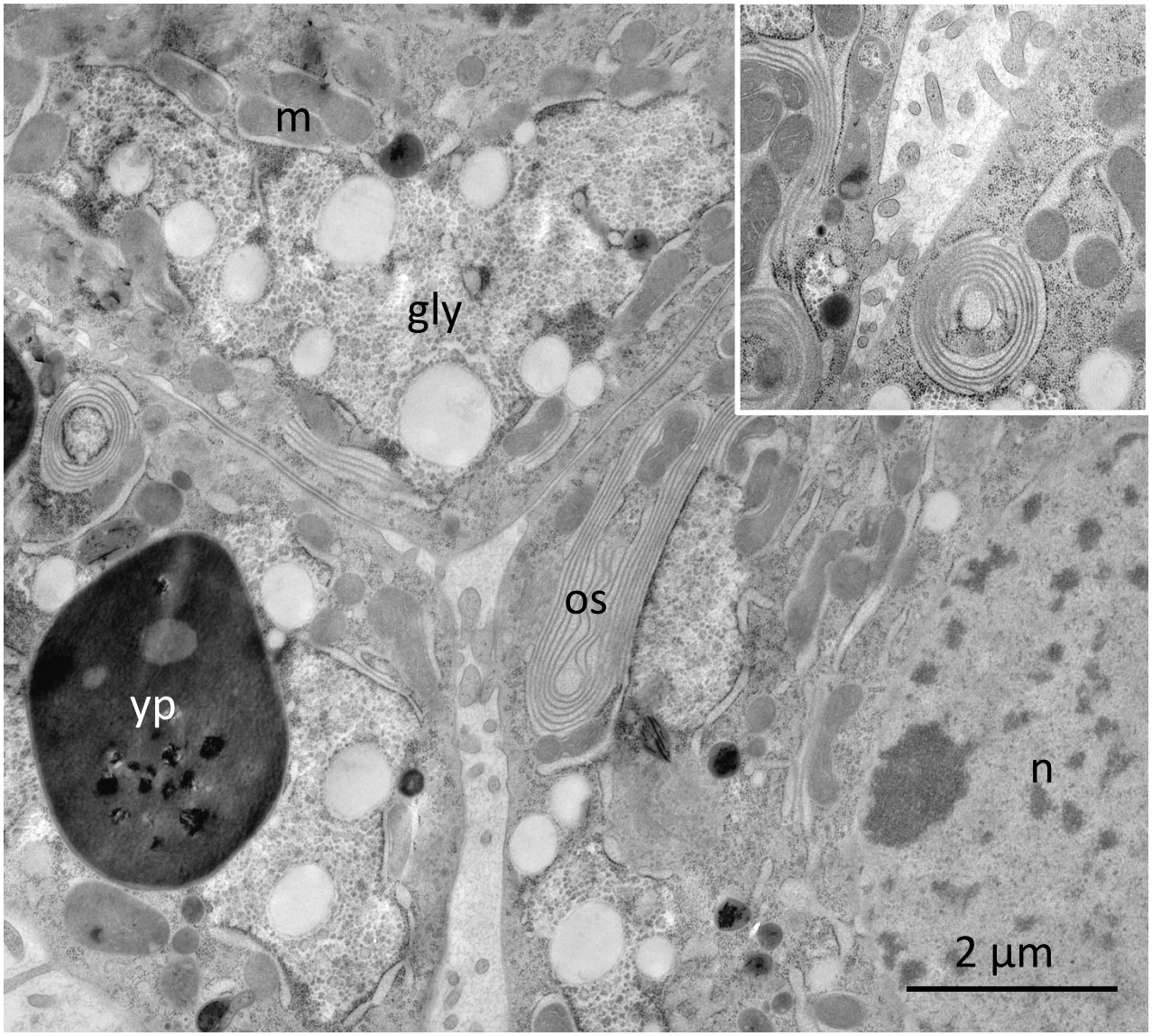

Fig. 29: Overview micrograph of three neighboring cells of the embryonic tissue located in the developing cyst. Extracellular space contains structures relevant for intercellular transport.(insert). Artifacts are lacking resulting in well prepared cell constituents. m, mitochondria; n, nucleus; gly, accumulations of glycogen; yp, yolk platelets.

Most prominent in the embryonic cells were numerous stacks and whorls of transformed ER in close association with mitochondria. Similar structures have been summarized previously under the term “organized smooth ER” (OSER) (Snapp et al. 2003). OSERs can be induced experimentally in tissue cultures by overexpression of specific proteins. In *A. franciscana*, we observed them regularly under physiological conditions as part of the embryogenesis. Notably, these OSERs exist in continuity with rER, since we found their outermost membranes decorated with ribosomes (Fig. 30).

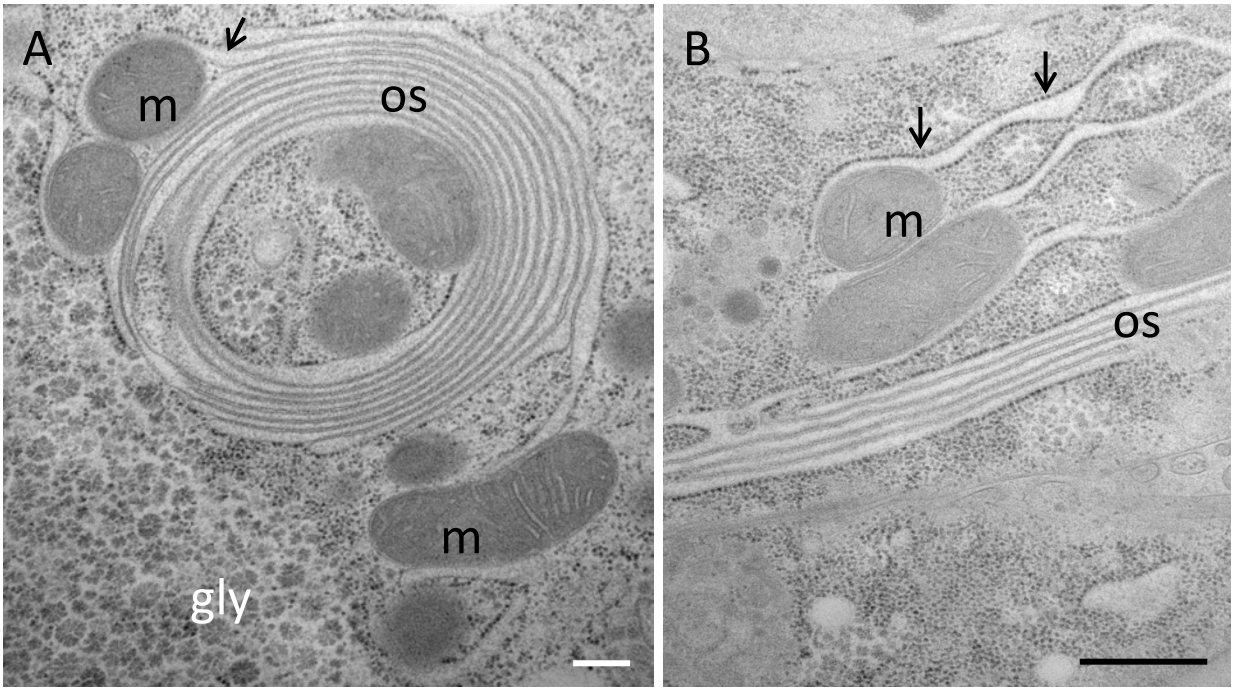

Fig. 30: Cryopreserved “OSERs” (os) in connection with mitochondria. A: Endoplasmic reticulum in a whirle state encircling mitochondria. B: ER organized as a stack in association with mitochondria that are aligned in parallel. Note that just the outermost ER layer is decorated with ribosomes (arrows).

3.2.7. Intact eggshell of rehydrated cysts hamper freeze substitution and resin infiltration

Fully developed eggshell layers of the cysts in diapause protect the embryonic cells inside and so their composition poses also a hindrance for proper cryopreparation. Nevertheless it was possible to preserve the embryonic cells of the rehydrated cysts by using HPF and AFS. In contrast to cysts with intact eggshells that were chemically fixed at room temperature, the cryopreparation gave the possibility to cut sections of the embedded material with the ultra-microtome, which could be evaluated in the TEM.

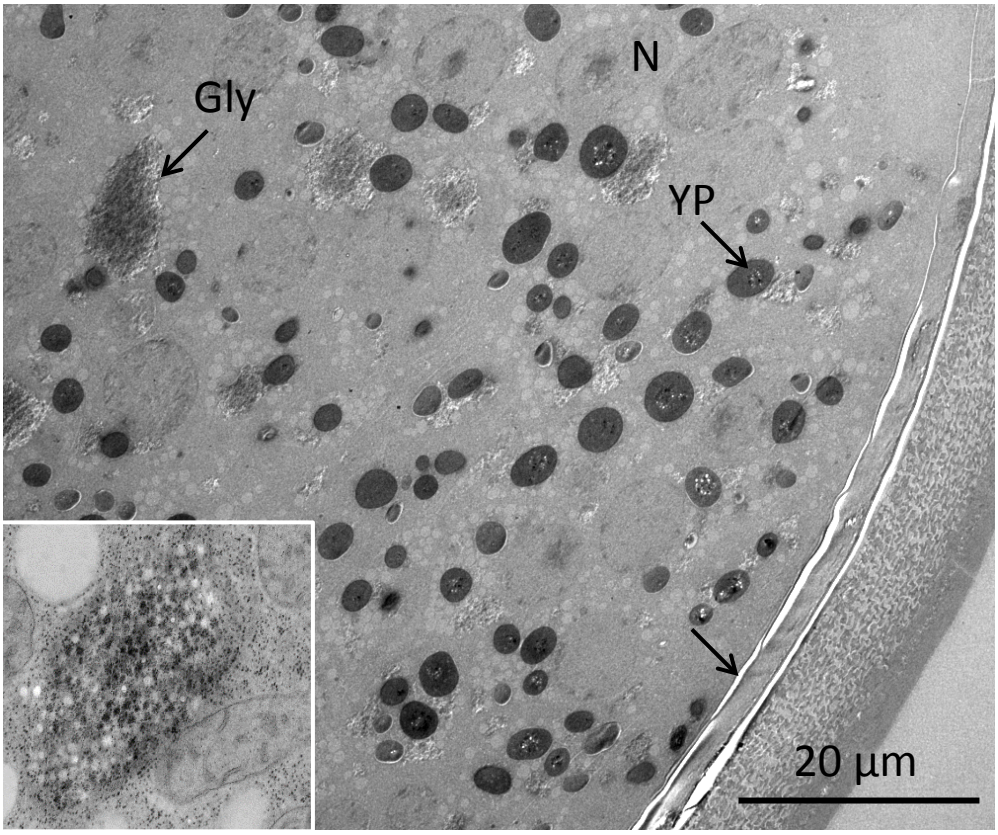

Fig. 31: Overview electron micrograph of rehydrated cysts with intact eggshell preserved by cryofixation. Embryonic cells contain properly preserved glycogen accumulations (Gly), yolk platelets (YP) and nuclei (N). Ice crystal formation in glycogen accumulations (insert) and disruption of the eggshell (arrow), caused by incomplete AFS and insufficient resin infiltration are clearly visible.

Application of HPF on rehydrated cysts, followed by FS under agitation for 9 hours resulted in proper cryofixation of the embryonic tissue. Problems, however, are indicated for both the FS and the resin embedding: i) The substitution of frozen cell water appears to be incomplete, resulting in ice crystal artifacts associated with glycogen stores, and ii) disruption of the interfaces related to the inner and outer embryonic cuticle (Fig. 31). Since resin infiltration was completed throughout the sample, tensions between the egg shell layers and the resin, related to insufficient fixation and/or embedding, appeared. In consequence, this resulted in the disruption during sectioning, which became more severe with increasing thickness of the section.

3.2.8. Preservation of the cysts with cracked eggshells

In contrast to eggs with intact shell layers, artificially broken eggshells, provide the best conditions for low-temperature fixation and resin embedding of the embryonic cells (Fig. 32).

A clear difference between processing results for closed and opened eggs (Figs. 32 and 33) was noticeable: i) The continuation of the shell layers, consisting of the outermost alveolar layer, the outer cuticle membrane, fibrous layer and a subsequent inner cuticle membrane (Ma et al., 2013) could be maintained if the eggshell was broken, and ii) the aspects of the embryonic tissue such as nuclei, mitochondria, yolk platelets were particularly well-preserved under these conditions. Nevertheless, there might be ice crystal formation in association with glycogen clusters, unless the substitution time is prolonged.

One important aspect of the cryopreservation is the maintaining of extracellular spaces (ex). Conventional processing at room temperature would result in extraction of the extracellular material and, perhaps, collapse of this space (Fig. 33).

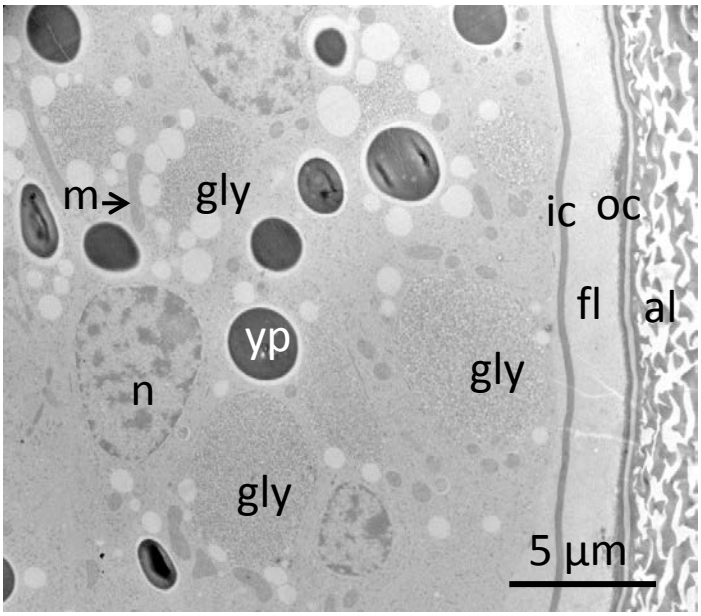

Fig. 32: Cryofixed rehydrated cyst with artificial disruption of the eggshell. Embryonic cells are well preserved: Yolk platelets (yp), nuclei (n) and mitochondria (m) appear in proper shape. Glycogen clusters (gly) are likely to form ice crystals, which indicate incomplete FS. Nevertheless eggshell layers appear clearly distinguishable: inner cuticle membrane (ic), fibrous layer (fl), outer cuticle membrane (oc) and the outermost alveolar layer (al).

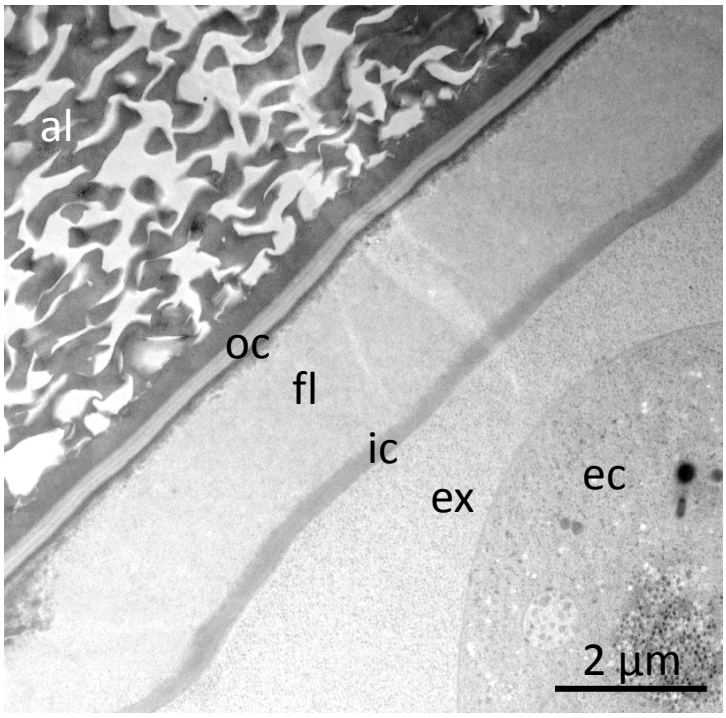

Fig. 33: Detailed micrograph of the different eggshell layers of the rehydrated cysts. The alveolar layer (al), three layered outer cuticle membrane (oc), the fibrous layer (fl) and the inner cuticle membrane (ic) represent the barrier protecting the cysts embryonic cells within (ec). Between the cells and the shell extracellular material is visible, which would be extracted using chemical fixation at room temperature.

4. Discussion

4.1. Chemical processing at room temperature versus cryopreparation

In this study differences in the ultrastructure of tissue and cysts of *A. franciscana* were documented. Cryofixation, namely high pressure freezing (HPF) and subsequent automatic freeze substitution (AFS) was compared with chemical fixation at room temperature with 4% PFA dissolved in PHEM buffer. Chemical fixation is widely used for morphological investigations and at its best it gives good ultrastructural preservation, but it is also known to cause typical artifacts like shrinkage of cells and organelles and extraction of cell components. Cryopreparation, helps to reduce such artifacts to a minimum.

Chemical fixation supported by microwave radiation gave results comparable with earlier studies (Zechmann & Zellnig 2009). The tissues of nauplii were fixed, using this method. The typical artifacts of chemical fixation occurred, like shrinkage and swelling of organelles, rearrangement of ribosomes and glycogen rosettes and extraction of cell components. The preservation quality varied between specimen and tissue depth. Nevertheless regions of adequate preservation could be found, which allowed morphological conclusions about the tissue. In contrast to these results, proper preservation of cysts, which were rehydrated in adequate hypersaline water, was not possible with this method.

Improvement of the protocols might be achieved by changing factors like exposure time in the microwave, adjusted osmolarity of the fixative, fitting to this animal living in hypersaline water, and the buffer or variating dehydration times of the ethanol series.

In contrast, fixation by using HPF and AFS brought advantages in handling and preparation: By immediate immobilization using HPF cells could be preserved next to their native structure, which has important consequences for the interpretation of the ultrastructure. Regulation of buffers, which represents one of the most important factors in producing well preserved tissue, is no longer necessary with low temperature methods. Dehydration happens continuously and has a minimal impact on the tissue at low temperatures. This avoids typical artifacts known from chemical fixation. Artifacts caused by cryofixation, like ice crystal formation, are easily interpretable. They are the result of improper handling and non-fitting AFS protocols.

Nevertheless our results indicate that these advantages are decisive for further progressing in ultrastructural studies of specific aspects of *A. franciscana*:

1. The spatial organization of small non-membrane bound structures, e. g. ribosomes and glycogen, becomes accessible for studies. In contrast to chemical preparation at room temperature, glycogen and ribosomes seem to be organized in clusters rather than to be redistributed within cells and tissues.

2. Significant progress can be expected in studying vitellogenesis, since the vesicular structures involved are particularly well-preserved. The roundish vesicles of the yolk ´nucleus´ and their content become clearly distinguishable (Criel, 1992). This offers new opportunities for exploring its supposed role in the yolk synthesis.

3. The data presented here indicate that cryopreparation is superior, if compared with conventional chemical preparation, if it comes to studies of the eggshell formation. Cryopreparation not only preserved the shell layers, but also the interface between embryonic cells and the eggshell in better quality.

4. Prospects have been improved for studying of the cuticle and its new formation, in the course of the molting cycle.

5. We identified “OSERs” (Snapp et al. 2003) as a specific form of the ER organization during embryogenesis.

6. Studies of the newly discovered crystal-like inclusions that were found in the ovisac epithelium require cryopreparation.

For comprehensive studies of both the oviparous and the ovoviviparous reproduction of *A. franciscana*, however, technical solutions are required which also enable processing of intact, rehydrated cysts (see next section).

4.2. Nature of the specimen decides about the time schedule of the FS

In similarity to conventional chemical fixation at room temperature, the FS substitution schedule has to be adjusted to the requirements of the tissues to be processed. We studied three sample types, which differ fundamentally in their resistance to the substitution process: i) tissue of the nauplii, ii) developing eggs inside the dissected ovisac, and iii) rehydrated cysts, as part of the project for "Prototype Development Prize 2015". We wanted to know whether sample agitation during FS could possibly help to overcome the natural barriers for the substitution process in shorter time than under static conditions.

Applications to specimens not related to the project (algae, paramecia, mosses, etc.) demonstrated that the use of the agitation module can shorten the FS substantially to 2-3 hours. Testing of such a short protocol indicated that is also appropriate for preservation of ovisac tissues including oocytes, but too short for the media exchange via developing eggshells. Therefore, we established a protocol that is sufficiently long, namely ten hours, to allow studies of the egg development and eggshell formation in the ovisac. We found out, that the time needed to complete the substitution process is determined by the preservation of glycogen clusters. Glycogen was identified as the cellular constituent, which complete the substitution process last. If the program was too short, water thought to be bound to glycogen at a certain ratio (Brittain & Geddes 1978) , gave rise to ice crystals. With our FS protocol for 10 hours, we seem to be very close to the limits of speeding up FS, since 9 hours FS turned out to be insufficient already. The fully developed eggshell of the cysts has proven as a barrier too strong to be overcome in such short time. Future experiments will find out, whether long-term FS over days, supported by agitation could become a means to expose the inside of cysts without jeopardizing the integrity of their eggshell.

4.3. Discovery of novel epidermal inclusion of the ovisac

The discovery of epidermal inclusions in the epithelium lining the ovisac was very surprising, since it turned out that the finding did not rely on advanced cryopreparation techniques. As demonstrated in Fig. 34 A, these inclusions are also visible in chemical preparation at room

temperature. Comparison with cryopreserved samples, however, indicates that a major characteristic is lost, if the preparation is done the conventional way. In cryopreparations these inclusion are presented as continuous ´zebra stripe´- like patterns that are formed as ´flakes´ (Fig. 34 B). Conventional preparation has lost much of the electron lucent material by extraction. These results in an appearance that can be described as rhomboids aligned in parallel and arranged in groups. Similar structures have been observed previously in a diversity of cells and tissues, and interpreted quickly as crystals (Fawcett 1981).

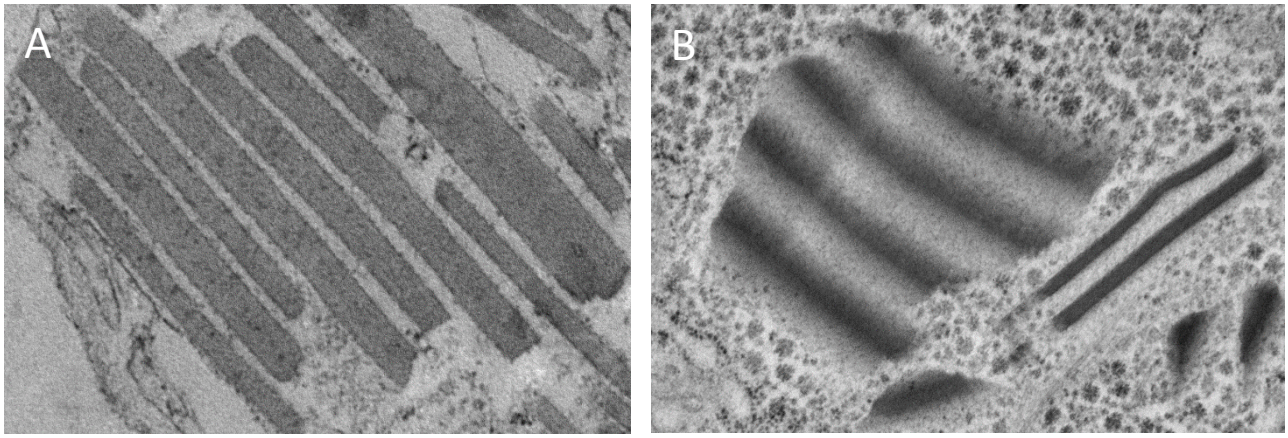

Fig. 34: Crystalline- like structures as epidermal inclusions. A: Chemically prepared ovisac results in great loss of the structure characteristics. B: Cryopreparation leads to ´zebra-stripe` pattern, formed as flakes.

In cooperation with other groups, we put efforts to elucidate the nature of the ´flake´-like inclusion in the ovisac epithelium. TEM studies at higher voltage (200 kV) in combination with electron tomography were applied by Prof. Neumüller (Medical University, Vienna). This approach aimed on the identification of grid-like crystal architecture at higher resolution. Electron diffraction (Dr. Stöger-Pollach, USTEM, TU Vienna) was performed in a search for diffraction patterns of crystalline origin. Despite of the overall crystalline appearance of the cellular inclusions, both methods did not provide evidence for their crystalline nature.

5. Abstract

The crustacean *Artemia franciscana* is able to switch between two different reproductive ways controlled by environmental cues: adult females can produce embryos in their ovisac, coated with a thin cuticle shell layer, ready to hatch immediately or metabolically inactive eggs, namely cysts, which undergo diapause in a late gastrula stage. These cysts can overcome desiccation of the habitat to reactivate and develop later, when rehydration is possible. For protection against harsh environmental conditions without water, the cysts are coated with a multilayered eggshell.

Scientists are attracted by the survival strategies of *A. franciscana*. They use this species as a model for studying egg development, which culminates in diapause. Transmission electron microscopy (TEM) has already provided insights concerning the internal and external morphology of reproductive organs, and processes such as fertilization, vitellogenesis and oogenesis. However, it cannot be overlooked, that the TEM data generated so far are overlaid by fixation problems and excessive extraction of biological material. Especially cysts, coated with a multilayered cuticle, hamper entrance of chemical fixatives and resin monomers commonly used for conventional TEM sample preparation.

Here, data are generated by a different approach of sample preparation for tissue of *A. franciscana* that is based on cryopreparation. The samples were processed by high-pressure freezing (HPF) and subsequent freeze substitution (FS) in OsO_4/ acetone and embedded in low viscosity resin. It is shown to what extent freezing techniques can help to preserve the ultrastructure of cells and tissues in involved in reproduction, like dissected ovisacs.

Furthermore, a possibility is demonstrated to accelerate the substitution process with overnight protocols, by application of an agitation module for automatized freeze substitution.

Although aimed at the cryoprocessing of *Artemia franciscana*, the approach chosen here could also be useful for ultrastructural studies of other specimens with similar resistance to conventional chemical sample preparation at room temperature.

6. Zusammenfassung

Artemia franciscana (Crustacea: Branchiopoda) kann, kontrolliert durch die aktuellen Umwelteinflüsse, zwischen zwei verschiedenen reproduktiven Wegen wählen. Das adulte Weibchen produziert bei günstigen Umständen Embryonen, die sofort nach der vollendeten Entwicklung zu schlüpfen (ovoviviparie), oder metabolisch inaktive Embryonen, namentlich „Cysten“, entwickeln sich, welche im Gastrula Stadium ihrer Entwicklung stoppen. Diese „Cysten“ können das saisonale Austrocknen ihres Habitats überleben, um sich dann wieder durch Wasserzufuhr weiterzuentwickeln. Durch eine vielschichtige Eischale sind sie vor aggressiven Umwelteinflüssen ohne Wasser geschützt.

Naturwissenschaftler sind fasziniert von den Überlebensstrategien von *Artemia franciscana*. Daher dient die Eientwicklung der „Cysten“ als gutes Beispiel zum Studium der Diapause. In früheren Studien wurde durch die Transmissionselektronenmikroskopie (TEM) über die Morphologie der Reproduktionsorgane und die Prozesse der Fertilisation, Vitellogenese und Oogenese berichtet. Dennoch sind bestehende Daten teilweise durch Fixierungsprobleme, wie Extraktion von Zellmaterial, behaftet. Vor allem die Dauereier der Krebstiere sind durch ihre dicke, vielschichtige Eischale schwer mit der konventionellen chemischen Methode für TEM zu fixieren, da sie das Eindringen der Fixative verhindert.

Durch einen neuen Versuch das Gewebe in einer besseren Qualität darzustellen, wurde in dieser Studie wurde das Gewebe von *Artemia franciscana* mittels Cryofixierung generiert. Proben wurden per „High Pressure Freezing“ (HPF) und anschließender „Automatic Freeze Substitution“ mit einer Osmium/ Aceton Mischung behandelt und anschließen in „Low Viscosity Resin“ eingebettet. Durch diese Methode konnte die Ultrastruktur der Zellen erhalten und typische Artefakte, die durch die chemische Fixierung bei Raumtemperatur entstehen, vermieden werden. Außerdem wurde auch die Beschleunigung des Substitutionsprozesses, durch die Zuführung eines Agitationsmoduls, ermittelt. Neben der Erforschung der Ultrastruktur von *A. franciscana*, kann die Methode der Cryofixierung auch für andere Proben, die ähnliche Probleme während der chemischen Fixierung aufweisen, verwendet werden.

7. Acknowledgements

Without these people my master thesis would not have been possible and a lot less great without their help and encouragement:

Waltraud Klepal: thank you for giving me the opportunity to write my master thesis in this department. I really enjoyed being a part of this research group and that you always had supportive and motivating words for me, even if something went wrong in my studies.

Siegfried Reipert: thank you for your patience and endurance, teaching me all skills related to chemical fixation and high pressure freezing procedures and giving me the freedom to explore what I was interested in.

Daniela Gruber: if I could I would write a special edition of acknowledgements only for you! Thank you for all the teaching and being the "patience in person" I every situation! Talking with you was always encouraging.

Marieluise Weidinger: thank you for being such a joyful person, it was fun working with you and be on the road to the congress in Hungary.

Norbert: thank you for your support and taking time for me, especially when I had troubles with the TEM! But also for the coffee- break talks.

Janek: thanks for being interested in my work and try to help me to carry on by supporting me with new ideas and suggestions.

Irene Lichtscheidl and Ingeborg Lang: thank you for supporting me in scientific questions, especially in relation to light microscopy. But also for the nice "come togethers", which made a super nice climate to work in our department, even if we all were spatially separated.

Stefan Sassmann and Sebastian Antreich: thank you for working with me and bringing a huge amount of fun into work! Special thanks to Stefan, who was always ready to make some light microscopical pictures of my *Artemias*.

Helmuth Goldammer: thank you for your technical skills- without them, Artemia rearing wouldn't be possible!! But also for supporting me with high quality light microscopy.

Thomas Schwaha: thank you for your time, doing so much confocal microscopy for us, supporting our next project and publications.

Daniel Abed- Navandi: thank you for the algae supply to feed the *Artemias*. Without you I wouldn't have the results I can present in my thesis now.

Sebastian: thank you for being there for me, no matter how exited or frustrated I was during my studies.

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9. Curriculum Vitae

Personal Data

Born August 11., 1988 in Vienna
Austrian citizenship

Education

Thesis on “Cryofixed Artemia: a new approach to better ultrastructure” at the Core Facility Cell Imaging and Ultrastructure Research (CIUS) since 2012

Master curriculum in *Behavior-, Neuro-, and Cognitionbiology*; University of Vienna since 2012

Bachelor in Biology with focus on Zoology at University of Vienna, 2008 to 2012

Matura degree 2008

Middle and High school at the *Sacre Coeur Pressbaum*

Work experience

Employee and collaborator within the Austrian Science Fund (FWF) - project of Dr. Siegfried Reipert; “Agitationmodul for Freezesubstitution” (PRIZE 2014 P1404894)

Tutor of “Submicroscopical anatomy and preparatory techiques in electron microscopy” at CIUS

Techinical Know- How

Transmission electron microscopy (Zeiss Libra 120 and 902)

Fixation techniques: chemical fixation incl. microwave fixation, cryofixation (Plunge and High pressure freezing; Automatic freeze substitution)

Epoxy resin and Lowicryl embeddings of cells and tissue

Ultrathin sectioning

Immunogold labeling for electron microscopy

Fluorescence and confocal microscopy

High resolution scanning electron microscopy (Philips XL 30 ESEM)

Critical point drying, carbon and sputter coating