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Molecular transfer to Atlantic salmon ovulated eggs using liposomes

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Abstract:

The delivery of exogenous biomolecules into teleost eggs is currently mostly relying on the manual microinjection methods, which, due to their high costs and low throughput, are not economically feasible for large-scale aquaculture applications. The main objective of this study was to develop a convenient, simple immersion delivery model for unfertilized Atlantic salmon (*Salmo salar* L.) eggs using liposomes as a system for the delivery of selected molecules.
We used a lipophilic marker, Rhodamine B isothiocyanate (RBITC) dye, to follow liposomal uptake, marker release and marker distribution within incubated egg. We investigated the influence of the incubation time, liposome surface charges and liposome sizes on the uptake into the eggs. After incubation with liposomes, the exposed eggs were fertilized and embryonic development was monitored until complete yolk sac absorption. The result showed that RBITC, was successfully delivered to the yolk of the incubated eggs. Moreover, during embryonic development, liposomal RBITC remained in the yolk sac until the yolk was completely absorbed. The findings demonstrated a novel approach for the delivery of exogenous molecules to unfertilized Atlantic salmon eggs, opening an avenue for large-scale aquaculture therapeutic applications.

Key words: delivery; liposomes; rhodamine B isothiocyanate; egg; Atlantic salmon

Highlights

1. Liposome mediated delivery of exogenous molecules to unfertilized egg is possible.

2. Documentation of distribution and localization of exogenous molecules in the embryo post fertilization.

3. This novel approach opens an avenue for large-scale aquaculture therapeutic applications.

1. Introduction

Aquaculture is currently among the fastest growing food-producing industries in the world (FAO, 2016). Within the aquaculture industry there is an obvious need for a large-scale delivery of bioactive molecules to eggs and developing embryos. For example, delivery of molecules that induce temporal or permanent changes in gene expression, improve embryonic nutritional status or prevent disease outbreak during the early stages of development, may all be beneficial for the industry both economically and regarding fish welfare issues. For decades, the delivery of exogenous biomolecules for manipulation of embryonic gene expression or gene transfer into fertilized teleost eggs has mostly relied on microinjections. Microinjection is a cumbrous, costly and time-consuming method and therefore not feasible for intensive aquaculture practices where thousands of eggs need to be treated at the same time (Szelei et al., 1994; Wong and Zohar, 2015). Thus, for aquaculture practices and uptake of biomolecules to large number of eggs, there is a need for more efficient, easy and reliable alternative delivery approaches.

Nanosystems are promising tools for efficient delivery of biomolecules in mammalian cell models. Among these, liposomes are the most common and extensively studied nano-carriers for targeted drug delivery (Chen et al., 2013; Sercombe et al., 2015). Key features of liposomes are their versatility, non-immunogenicity and non-toxicity (Akbarzadeh et al., 2013; Allen and Cullis, 2013; Laouini et al., 2012; Schwendener, 2014). The cargo molecules, depending on their chemical properties, can be included in different compartments of liposomes: water-soluble compounds (proteins, peptides, nucleic acids, carbohydrates, haptens) are entrapped within the aqueous inner space of liposomes, whereas lipophilic compounds (drugs, lipopeptides, adjuvants, linker molecules) are intercalated into the lipid bilayer. Such dual transport capacity opens up the possibility for efficient entrapment of a wide variety of molecules. Moreover, the versatility of such carrier systems (Sercombe et al., 2015) increases the safety and efficacy of administration (Shi et al., 2010). However, in aquaculture, use of liposome-based delivery is still in its infancy compared to mammalian application.

In aquaculture, liposomes have been, up to now, mostly used as carriers for zooplankton nutrients enrichment (Barr and Helland, 2007; Hawkyard et al., 2015) in bivalve mollusc larvae (*Venerupis decussatus* and *Venerupis pullastra*) (Lai et al., 2004) and therapeutic agents, such as immunostimulants and vaccines (Ji et al., 2015; Ruyra et al., 2013). For example, liposomes with lipopolysaccharides (LPS) from *Aeromonas salmonicida* gave rainbow trout better protection against furunculosis than the LPS administered alone (Nakhla et al., 1997). However, very few studies have demonstrated delivery of molecules to fish fertilized eggs or zygotes in large-scale. Such scarcity is likely due to high protection of the fertilized eggs provided by the chorion, hence, a method circumventing this limitation would be beneficial for the aquaculture industry.

In salmonids, ovulated eggs can be stored in celomic fluid or balanced salt solutions for an extended period without any significant loss of egg quality (fertilization and developmental capacity). Storage time is dependent on the temperature and media composition (Niksirat et al. 2007). In rainbow trout, this "ovulation window" was reported to be up to nine days when eggs were stored in a coelomic fluid at 2-3 °C (Niksirat et al., 2007) or at least two days at 12-13 °C, when stored in a modified Cortland solutions (Goetz and Coffman, 2000). Moreover, all teleost eggs are surrounded by an extracellular protective outer envelope called zona radiata or chorion, covering the egg plasma membrane (Yamagami et al., 1992). Ultrastructure examination of the salmonid chorion shows numerous pore channels (Schmehl and Graham, 1987), which may potentially allow passage of biomolecules. After fertilization and egg activation, rapid changes in the chorion structure occur, which might limit chorion passage for larger molecules. Fertilized eggs in coho salmon have been found to take up lipophilic molecules of low molecular weight (e.g. sex steroids) *ex vivo* (Piferrer and Donaldson, 1994). Whether the egg chorion will allow passage of different charged molecules such as proteins or even nano-carriers, still needs to be investigated. The aim of the present work was to explore the permeability of ovulated eggs for molecular delivery. We hypothesized that utilizing the unique features of the egg during the "ovulation window" would make it the most convenient and safe period for delivering biomolecules to salmon egg for large-scale aquaculture applications.

Here, we developed a method for the *in vitro* transport of lipophilic biomolecules into unfertilized salmon eggs using incubation with liposomes of various sizes as a delivery carrier. *In vivo* bio-distribution in the fertilized eggs and developing embryos were further explored. The liposomal uptake across the chorion and through the egg plasma membrane, and distribution within the egg was monitored using Rhodamine B isothiocyanate (RBITC) as an

imaging molecule. RBITC is a well-established lipophilic dye (Xiong et al., 2010). It is known to be non-toxic to immature neurons, permitting the dye-filled axons to continue their growth towards their targets within the CNS. Especially interesting for us was the fact that this dye does not leak from the labeled axons or cells (Thanos et al., 1987). Due to its lipophilicity, it is expected that it will accommodate itself within the lipid bilayers of liposomes. Furthermore, the influence of liposomal size and surface charge on the uptake efficiency was evaluated.

2. Materials and methods

2.1. Materials

Lipoid S 100 (PC, soybean lecithin, >94% phosphatidylcholine) and egg phosphatidylglycerol sodium Lipoid EPG-Na (PG) were obtained from Lipoid (Ludwigshafen, Germany), Rhodamine B isothiocyanate (RBITC), cholesterol (Ch), sodium hydroxide (NaOH), Leibovitz (L-15) medium powder with glutamine, penicillin, streptomycin, HEPES, TES, were purchased from Life Science (Sigma–Aldrich Norway AS, Oslo). Methanol was obtained from Merck (Darmstadt, Germany). Dulbecco's phosphate-buffered saline (PBS, pH 7.4) and were obtained from Life Technologies (Paisley, Scotland). All used chemicals were of analytical grade.

2.2. Preparation of liposomes

Conventional liposomes containing RBITC were prepared by the film hydration method (Naderkhani et al., 2014). PC and cholesterol (9:1, w/w ratio; 495 mg total lipid) or PC, PG and cholesterol (9:1:1, w/w ratio; 540 mg total lipid) were dissolved without or with RBITC (10 mg) in methanol. Organic solvent was removed under vacuum using rotoevaporator system (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland) for at least 2 h at 50 mm Hg and 45 °C. The lipid films were hydrated with 10 mL of PBS (pH 7.4) to form the liposomal dispersions, namely control-PC/Ch (empty), RBITC-PC/Ch and RBITC-PC/PG/Ch. Liposomal suspensions were stored in the refrigerator (4–8 °C) overnight prior to further use.

2.3. Characterization of liposomes

Liposomes (5 mL; 49.5 mg/mL or 54.0 mg/mL, respectively) from each formulation was transferred to 10 mL beaker and placed on ice bath. The sonicator (Ultrasonic processor 500 W, Sigma–Aldrich, St. Louis, MO, USA) was set to 40% amplitude and the liposomes were exposed to ultrasonic irradiation for 1, 2 or 2 × 2 min, respectively, to obtain large and small

liposomes of approximately 160 nm and 50 nm in diameter, respectively. The sonicated liposomes were stored in the refrigerator for at least 6 h prior to further use. The free RBITC (Mw 536.08) was separated from liposomally entrapped RBITC by using Nanosep 100K (MWCO 100 KDa) modified polyether sulphone ultrafiltration devices (Pall life Sc, NY, USA). The supernatant, liposomal suspension free of unentrapped RBITC, was used for further characterization. The particle size distributions of liposomes were determined by photon correlation spectroscopy (PCS) (Submicron particle sizer model 370, Nicomp, Santa Barbara, CA, USA) and zeta potential measurements were performed on a Malvern Zetasizer Nano Z (Malvern, Oxford, UK) as described according to Jøraholmen et al. (Jøraholmen et al., 2015). The liposomes were stored at 4 °C and used in experiments within 7 days after preparation.

2.4. Morphology of liposomes: Transmission electron microscopy (TEM) observation

Transmission electron microscopy (TEM) imaging was used to observe the vesicle morphology (lamellarity) of liposomes after size reduction. TEM images were recorded by using a Jeol (Tokyo, Japan) Model 1011 transmission electron microscope operating at 80 kV. To prepare the TEM samples, 5 µL liposomal suspension was dropped onto a carbon-coated copper grid directly from solution and dried prior to observation.

2.5. *Ex vivo* uptake of liposomal RBITC

In vitro culture of ovulated eggs

A modified version of the L-15 culture media with slight variation from the trout media (Nagler et al., 1994) was used for the *in vitro* culture of ovulated eggs. The modified L-15 medium was adjusted to 300 mOsm with distilled water and filtered using a 0.22-µm pore-size filter and the pH was adjust to 7.7 using 1.0 N NaOH. This medium maintained the eggs in healthy condition for at least 5 days (own observation). The temperature for maintenance of the unfertilized salmon eggs and milt was 2-4 °C. Fresh ovulated eggs and milt of sexually mature (3-4 years) Atlantic salmon were obtained from the breeding company AquaGen, Trondheim, Norway, by an overnight transport (2 °C) to Nofima, Tromsø. Before starting the experiment, eggs were washed thrice with PBS (pH 7.4) and subsequently washed twice with modified L-15 medium. Throughout the experiments, the freshly prepared buffer and medium was maintained at 4 °C. The experiments/procedures described herein have been conducted in accordance with the laws and regulations controlling experiments/procedures for live animals in Norway, i.e. the Animal

Welfare Act of December 20th 1974, No 73, chapter VI sections 20-22 and the Regulation on Animal Experimentation of January 15th 1996.

Uptake studies

Washed eggs, all from the same batch, were distributed (100 eggs/petri dish) in a petri dish containing 30 mL of modified L-15 medium. The experiment was conducted in duplicate. Liposomes (1.5 mL, 49.5 or 54.0 mg/mL) representative of different groups including the controls (Table 1) were incubated with the unfertilized egg. After 24 and 48 h of incubation at 4 °C with continuous shaking at 45 rpm, the ovulated eggs were washed thrice with PBS to remove the traces of attached liposomes, followed by fertilization with salmon sperm in excess. Briefly, 100 µL of sperm was added to the washed eggs in the beaker. Both eggs and sperm were mixed by adding 20 mL of de-chlorinated water, and incubated for 1 min. Then, more water (200 ml) was added slowly to the beaker without disturbing the eggs and left undisturbed for minimum 4 h. The eggs were washed slowly with fresh water and transferred to the 8-tray egg incubator where they were kept in the dark with running water at 7 °C until hatching. Dead eggs or embryos were removed from holders every other day. At least 10 eggs/embryos were sampled for evaluation of internalized RBTIC-containing liposomes by Zeiss fluorescence microscope at different developmental stages (ovulated eggs and at 196, 490, 700, 840 day-degrees, DD). All the settings and exposure time were kept constant for all treatments including controls. The ovulated eggs or the fertilized eggs were punctured to collect either yolk or the embryo with or without yolk sac for further analysis under fluorescence microscope, to examine the uptake of the RBITC-containing liposomes into the yolk and RBITC delivery to the embryos. The ovulated eggs post 24 h and 48 h of incubation with different liposome groups were washed with PBS and fixed in 4 % paraformaldehyde (PFA) for confocal microscopy.

3. Results

3.1. Characterization of liposomes

The different liposomal formulations were grouped according to the particle size, size distributions and zeta potentials. Two targeted liposomal suspensions were prepared: the small liposomes with an average diameter of around 50 nm, and large liposomes with an average diameter of around 160 nm, respectively. Both types of liposomes were prepared as neutral and negatively charged liposomes, as confirmed by the zeta potential measurements (Table 1).

Vesicle morphology was confirmed by TEM. All liposomes were unilamellar in structure and approximately of size corresponding to the size measured by PCS (Fig. 1).

3.2. *Ex vivo* uptake of liposomal RBITC

Unfertilized salmon eggs were incubated in modified L-15 culture medium containing liposomes from the respective group. The presence of the red fluorescence, RBITC, in the yolk of unfertilized eggs incubated for 24 h and 48 h with liposomes was indicative of successful uptake/delivery of liposomal RBITC (Fig. 2). The uptake was not influenced by the size or the surface charge of the liposomes (Fig. 2). The result showed that 24 h incubation was sufficient for the RBITC-containing liposomes to cross the chorion and the egg plasma membrane and go into yolk. Confocal microscopy of the cryo-sectioned egg also confirmed the presence of liposomal RBITC in the yolk passing the chorion (Fig. 3). As 24 h incubation period showed to be sufficient for the liposomal RBITC to reach the yolk, we used this group in all further post fertilization studies. Moreover, there was 100 % survival of the unfertilized eggs after liposome treatment confirming the safe method of delivery.

3.3. Distribution of liposomal RBITC post fertilization and embryogenesis

To follow the systemic distribution of the lipophilic dye in embryos, the eggs pre-treated with liposomes were fertilized. The fertilization rate of pre-treated eggs showed to be similar to those of un-treated eggs, thus indicating that the exposure to liposomal RBITC did not affected the fertilization efficiency. Moreover, no developmental arrest or deformities in the liposome treated groups were detected as compared to un-treated controls, excluding the toxic or negative effects of the RBITC-liposomes on the embryonic development.

At developmental stage 196 day-degrees (DD), the chorion exhibited only traces of fluorescence and the vast majority of the fluorescent dye was restricted within the yolk. Traces of fluorescence were also visible in the intestine of the embryos. Similarly, at 490 DD, the fluorescent RBITC was detected only in the yolk sac and intestine of the embryo in all treatment groups (Fig. 4). The distribution of the fluorescent molecules persisted also at 700 DD of development, i.e. about 200 DD after hatching (Fig. 5). All the RBITC-liposomal groups exhibited very similar fluorescence levels during the corresponding stages. The eggs treated with empty liposomes did not exhibit any fluorescence and the level of auto-fluorescence was negligible. The fluorescence was maintained in the yolk until all yolk was absorbed; about one

and half month after hatching (840 DD post fertilization) which corresponds to the time of first exogenous feeding (Fig. 6).

4. Discussion

The main aim of the study was to develop a simple and user-friendly method for large-scale delivery of targeted molecules to unfertilized Atlantic salmon eggs. To explore the feasibility of such a method, we prepared conventional liposomes with the lipophilic dye (RBITC) and followed liposomal uptake, intracellular distribution and localization of the dye in the incubated eggs, and subsequently, embryos of Atlantic salmon.

The approach demonstrated a feasible method to deliver targeted molecules by utilizing liposomal carriers. To the best of our knowledge, it is the first time that such delivery has been implemented via incubation of ovulated salmon eggs.

The limitations of conventional microinjection method in teleost eggs are urging the fast development of novel methods able to deliver successfully the material of interests in the eggs of various fish species (Hostetler et al., 2003; Soroldoni et al., 2009; Szelei et al., 1994). Some of the attempts to overcome the limitations were proposed relatively early by Szelei et al. (Szelei et al., 1994) who explored the use of liposomes for plasmid delivery in the fertilized egg of African catfish as large-scale approach. Major limitation that hampered further application of this method was the dechlorination of the fertilized eggs. The present study, in contrast, focuses on the features of the unfertilized eggs to develop a more practical and convenient approach for the delivery of biomolecules.

The results of our study indicate that, under the present experimental conditions, an incubation window of at least 48 h has been established permitting safe liposomal delivery to Atlantic salmon eggs. To improve the efficiency of liposomal delivery, it is crucial to understand the mechanism of liposomal uptake by the cell and to follow the intracellular fate of internalized liposomes and their contents (Düzgüneş and Nir, 1999). The exact mechanism of liposomal uptake by the ovulated egg is currently unknown. At present, we attempted to investigate the uptake process in ovulated eggs by following the fate of liposomal RBITC. Our results indicate that, the liposomes were taken up, and the uptake was not affected by the liposomal size. Therefore, we speculate that the liposomes in the size range used here (50-160 nm) may have passed through the chorion pore channels to the egg plasma membrane. This conclusion is supported by previous chorion ultrastructure studies performed in salmonids (Schmehl and

Graham, 1987), in addition to our own observations, suggesting that the pore canal diameter is in size range of 200-500 nm. Moreover, we wanted to determine any possible effect of liposomal size within the size range below the pore size, as we postulated that smaller liposomes might be taken up to greater extent than the larger ones. However, as the size did not affect the uptake efficacy, the exact mechanism of the uptake remains not fully understood. One of the possible mechanism allowing direct transport over the pores could be "slipping" of the vesicles through the pores due to their size or pinocytosis (Peschka-Süss and Ŝkalko-Basnet, 2000) . In addition, Figure 3 shows a strong signal in the chorion as well as in yolk of the unfertilized eggs. To elaborate on this, the uptake through the chorion is a continuous process through pore channels within the chorion across the egg surface. The photographs presented here represent a particular time point of the uptake process, the moment it get fixed during sampling and it is, therefore expected that RBITC-containing liposomes may still be within the chorion when the sampling took place. Thus, the chorion will also show a red signal. We cannot also ignore the fact that during the uptake process, some rhodamine might become incorporated in chorion. Therefore, we speculate the red signal in the chorion is due to the ongoing uptake of rhodamine present during the fixation time as well as some incorporated rhodamine in the chorion. Both possibilities confirm, the appearance of a strong signal in the yolk sac and indicates the delivery of liposomes and (or) RBITC to the egg. Further studies, for example with hydrophilic dyes or larger liposomes (> 500 nm), could provide deeper insights on the uptake mechanism.

Several studies in higher vertebrates have shown that liposome surface charge has a profound effect on the liposome-cell interaction *in vitro* (Cho et al., 2009; Düzgüneş and Nir, 1999; Miller et al., 1998). Liposomal surface charge and applied concentrations have an effect on the liposome-cell interaction as well as the cytotoxicity as confirmed in the teleost (zebra fish and trout) studies (Romøren et al., 2005; Ruyra et al., 2013). Based on the similar findings regarding cytotoxicity of positively charged liposomes in fish and mammals, only neutral and negatively charged liposomes were used in the present research focused on the preliminary uptake study. Moreover, Szelei et al. (Szelei et al., 1994) reported the successful uptake of large negatively charged liposomes in African catfish zygotes at the 2-4 cell stage without providing any information on the exact size of liposomes and the uptake mechanisms. In contrast, the uptake of RBITC-containing liposomes was neither affected by the neutral or negatively charged liposomal surface. Further studies including positively charged liposome at lower dose are required to outline the exact uptake mechanism in teleost eggs.

The present study demonstrated that during the post-fertilization period and embryogenesis, the liposomal RBITC was localized in the lipophilic region of the embryo i.e. in the yolk sac, which serves as an energy substrate for the developing embryo (Heming and Buddington, 1988). Further, the dye was retained in the yolk and, subsequently, in the intestines of the developing embryos. The dynamics of dye extrusion implies that it was slowly metabolized by the embryo from the yolk sac reservoir, ensuring slow and sustained release of the model lipophilic compound within the embryo until the yolk sac was absorbed. Such a model opens up an avenue for the future applications with delivery and continuous release of lipophilic drugs, essential nutrients or other biomolecules in the fish embryo.

Moreover, in unfertilized eggs, we observed no mortality due to exposure to liposomes. Dead or developmentally compromised oocytes and eggs are easily recorded by their appearance (dead or unfertilizable eggs has typically a translucent ring in the periphery of the ooplasm) (Tyler et al., 1990); own observations). Also, close to 100% of the eggs were actually fertilized and developed normally. Thus, we are confident that, under the experimental conditions used here, this is a safe method for egg incubation.

Production of fish larvae is often hampered by high mortality rates and where some of these losses may be related to the nutritional quality of the food and low protection against infections due to a poorly developed immune system during the early stages of development. Liposome mediated nutrient supplementation to fish larvae has been applied either directly or indirectly by feeding liposomes to live food organisms which are then fed to fish larvae (Barr and Helland, 2007). However, such treatment will only be efficient after exogenous feed intake has started and the yolk sac has been absorbed. Thus, during embryogenesis, probably the most important period of an organism development, when most important organs and neuroendocrine axis develop, it is not possible to alter the nutritional composition of the developing fish by larval feeding. For example, A. salmon grow and develop for about four months (at 8 °C), relying only on nutrition provided by the yolk sac, before exogenous feeding is initiated. Today, changes in egg nutritional composition have to be induced by long (several months) and expensive broodstock feeding. Therefore, we suggest that liposome-mediated delivery of important yolk sac components to eggs may be an easier and more cost effective method for changing the egg nutrient composition for more robust larvae. Thus, this method may be a safe means to, in a controlled manner, investigate and improve the nutritional quality of egg and larvae. Further, our method, using RBITC as a model molecule, can be likely used for the delivery or uptake of small lipophilic substances such as essential fatty acids (EFA), and fat-

soluble vitamins (A, D, and E) as well as phospholipids, all being important for the growth and survival during the early stages of development. This method may also have the potential to deliver a variety of other low molecular weight hydrophilic substances, e.g. free-amino acids, water-soluble vitamins, minerals and oligonucleotides incorporated into the liposome core. Furthermore, considering the recurring problem of infectious diseases and high mortality rates during the early stages of fish development, which are probably related to a poorly developed immune system at these stages, liposome mediated delivery of pharmaceuticals or immuno-protectants to the egg/yolk may be a solution to mitigate these challenges.

Finally, the potential of this delivery approach based on liposomes to deliver also larger hydrophobic as well as hydrophilic molecules to fish eggs and their potential influence on embryonic development remain to be determined.

Conclusion

The study demonstrates that the liposomal delivery could be a powerful and safe tool for transmembrane delivery of selected molecules to salmon eggs. The approach highlights the advantage of liposomes over individual microinjections, a pro for large-scale applications in salmon aquaculture. Moreover, this approach could be successfully adapted to other fish species and easily modified for liposomal delivery of lipophilic biomolecules, essential nutrients or hydrophilic biomolecules, such as oligonucleotides, for targeted manipulation of gene expression.

Conflict of interest

The authors report no conflicts of interest in this work.

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Table

Table 1. Experimental setup and liposome characteristics. The data are expressed as mean ± S.D. (n=3)

Experimental groups	Types of liposomes	Mean diameter (nm)	Polydispersity Index (PI)	Zeta (ζ) potential (mV)
Group A	Control (empty liposomes/only media)	75.2 ± 45.6	0.36	-1.77 ± 5.6
Group B	Small neutral RBITC-liposomes	55.6 ± 35.1	0.36	-1.16 ± 4.8
Group C	Large neutral RBITC-liposomes	161 ± 90.4	0.32	1.026 ± 4.9
Group D	Small negatively charged RBITC-liposomes	53.6 ± 33.0	0.27	-26.57 ± 6.0
Group E	Large negatively charged RBITC-liposomes	166.5 ± 57.0	0.32	-33.23 ± 4.52

Figure captions

Fig. 1. Liposome morphology. Representative transmission electron microscopy (TEM) image of PC/cholesterol liposome showing the morphology and architecture of the empty liposomes. Scale bar = 500 nm

Fig. 2. RBITC-liposome uptake in the ovulated eggs. Uptake of RBITC-liposomes in the ovulated eggs after 24 and 48 h of incubation. The control group (Gr. A) did not exhibited any fluorescence. Fluorescence was analyzed in the extracted yolk using a Zeiss fluorescence microscope. Details of group are provided in Table 1. Scale bar = 2 mm

Fig. 3. Confocal laser-scanning microscopic images of Atlantic salmon unfertilized eggs after 24 h and 48 h incubation with empty and RBITC-liposomes. Confocal microscopy was performed using a Leica TCS SP5 confocal microscope (Leica Microsystems Gmbh, Mannheim, Germany). Panels represents overlays of the fluorescent image onto phase-contrast images. The excitation wavelengths for RBITC used was 561 nm. All images were generated with 20 x 0.4 NA dry objective lens using the same settings. Parts of the sectioned ovulated egg were labelled: Ch, chorion; Y, yolk.

Fig. 4. RBITC distribution and localization in the embryos after incubation with the ovulated eggs. RBITC localization in the yolk sac of embryos at 490 DD (70 days post fertilization) and also in the intestine (yellow arrow head) after 24 h of incubation with RBITC-liposomes. Scale bar = 2 mm

Fig. 5. RBITC distribution and stability in the embryos after 24 h incubation with the ovulated eggs. RBITC localization in the yolk sac of hatched larvae at 700 DD (100 days post fertilization) and in the intestine. Scale bar = 2 mm

Fig. 6. Localization of RBITC just after yolk sac absorption. Traces of RBITC found near the yolk sac (shown by arrow) in the RBITC-liposome treated group even after yolk sac absorption i.e. after 1.5 months of hatching (120 days post fertilization). Control group showed no fluorescence. Scale bar = 2 mm

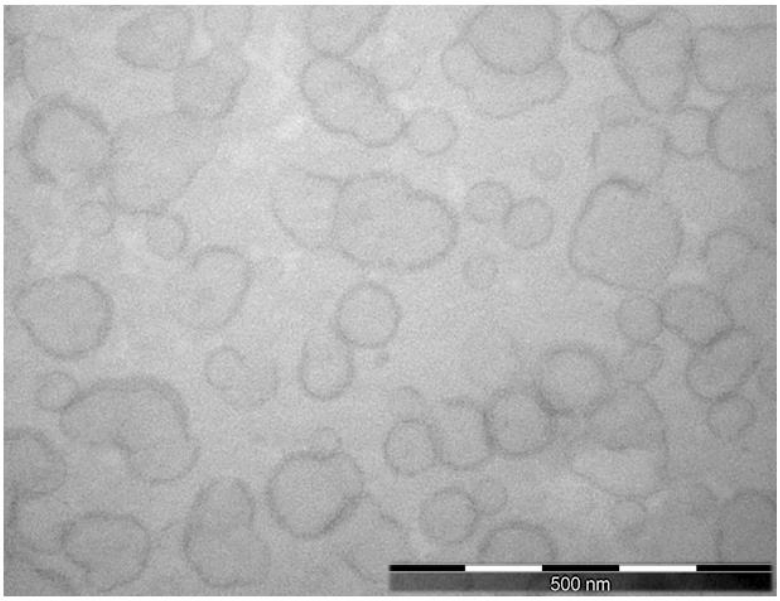

Figure 1

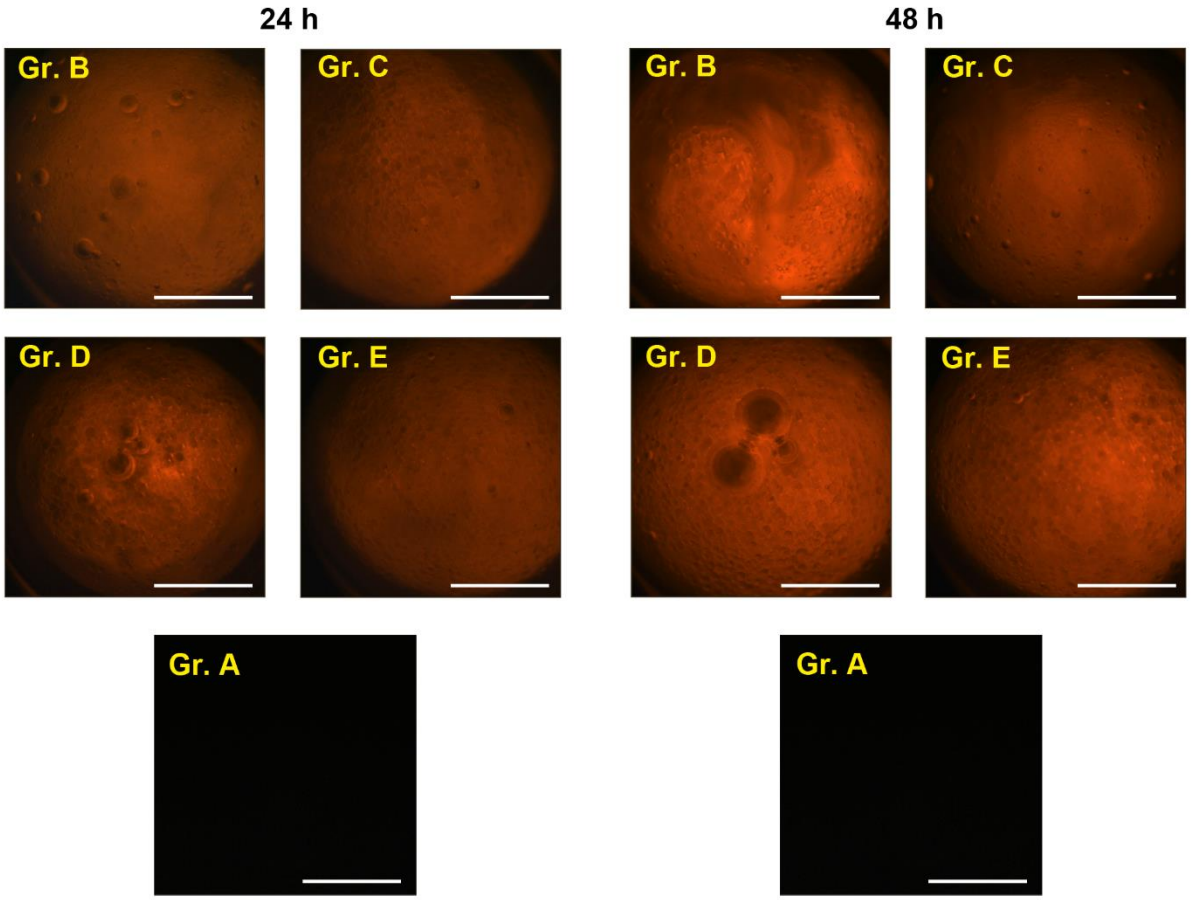

Figure 2

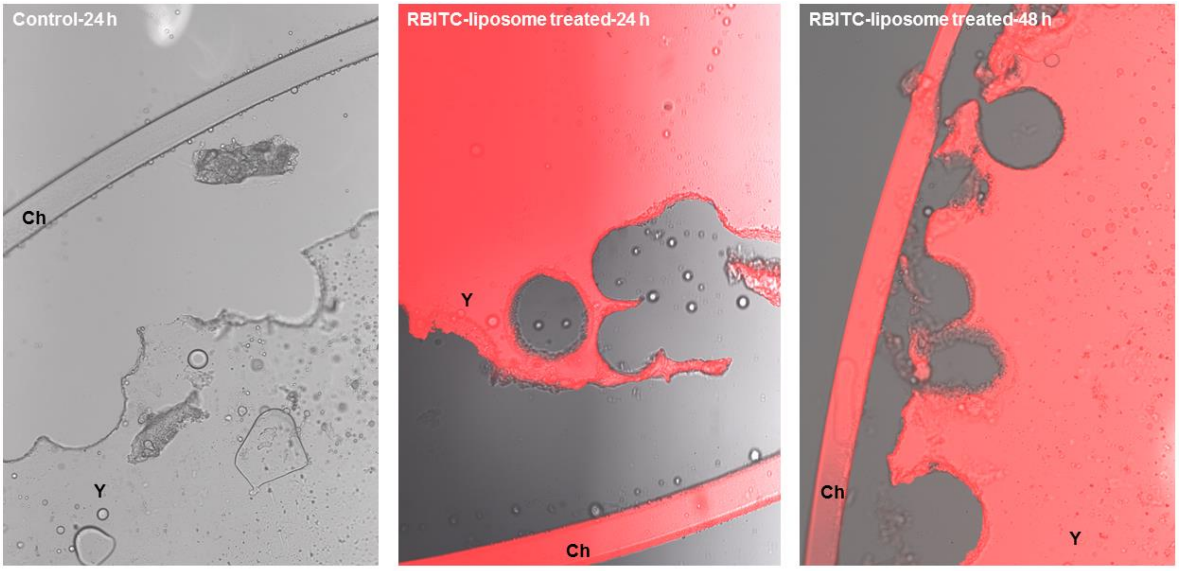

Figure 3

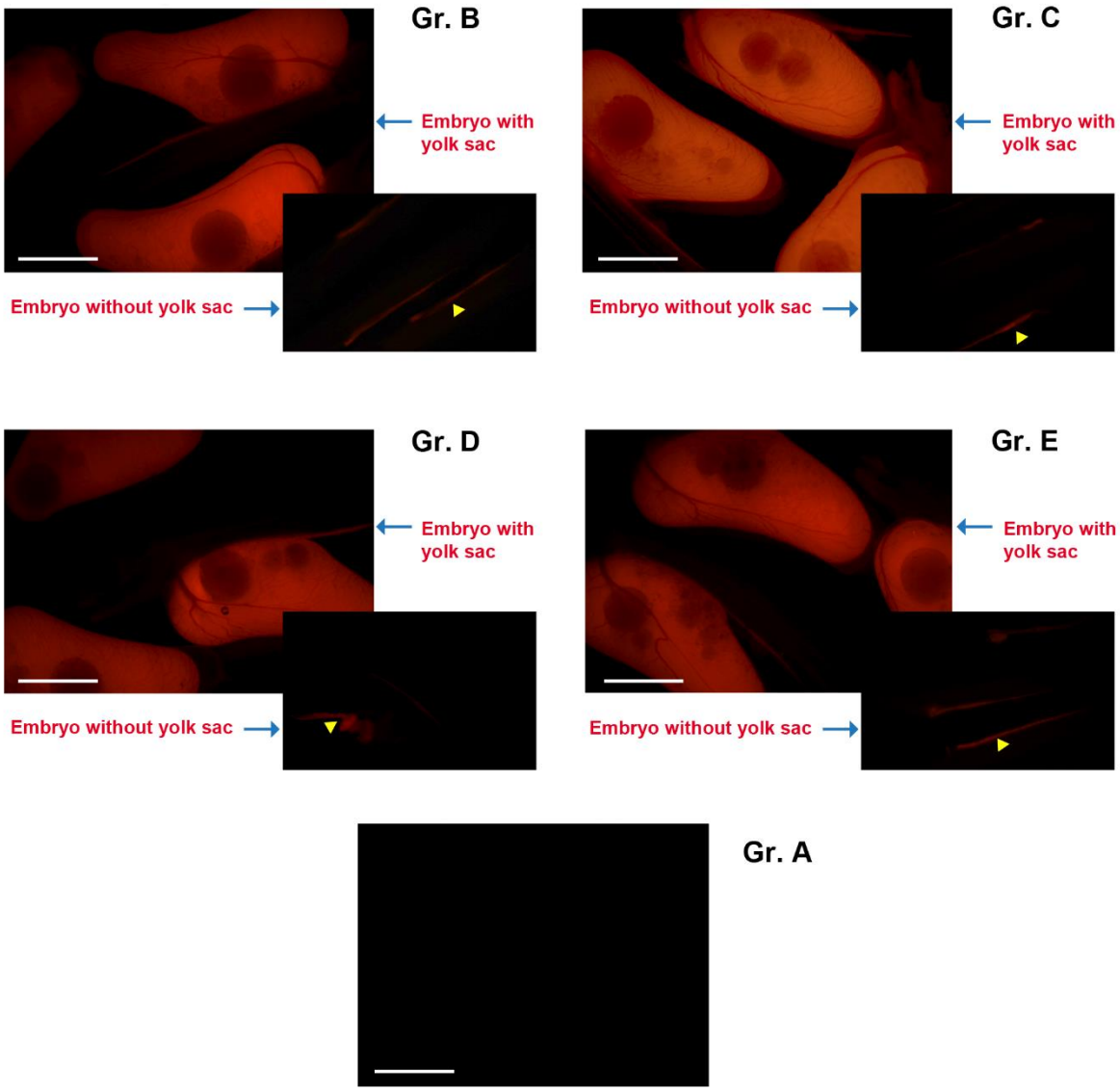

Figure 4

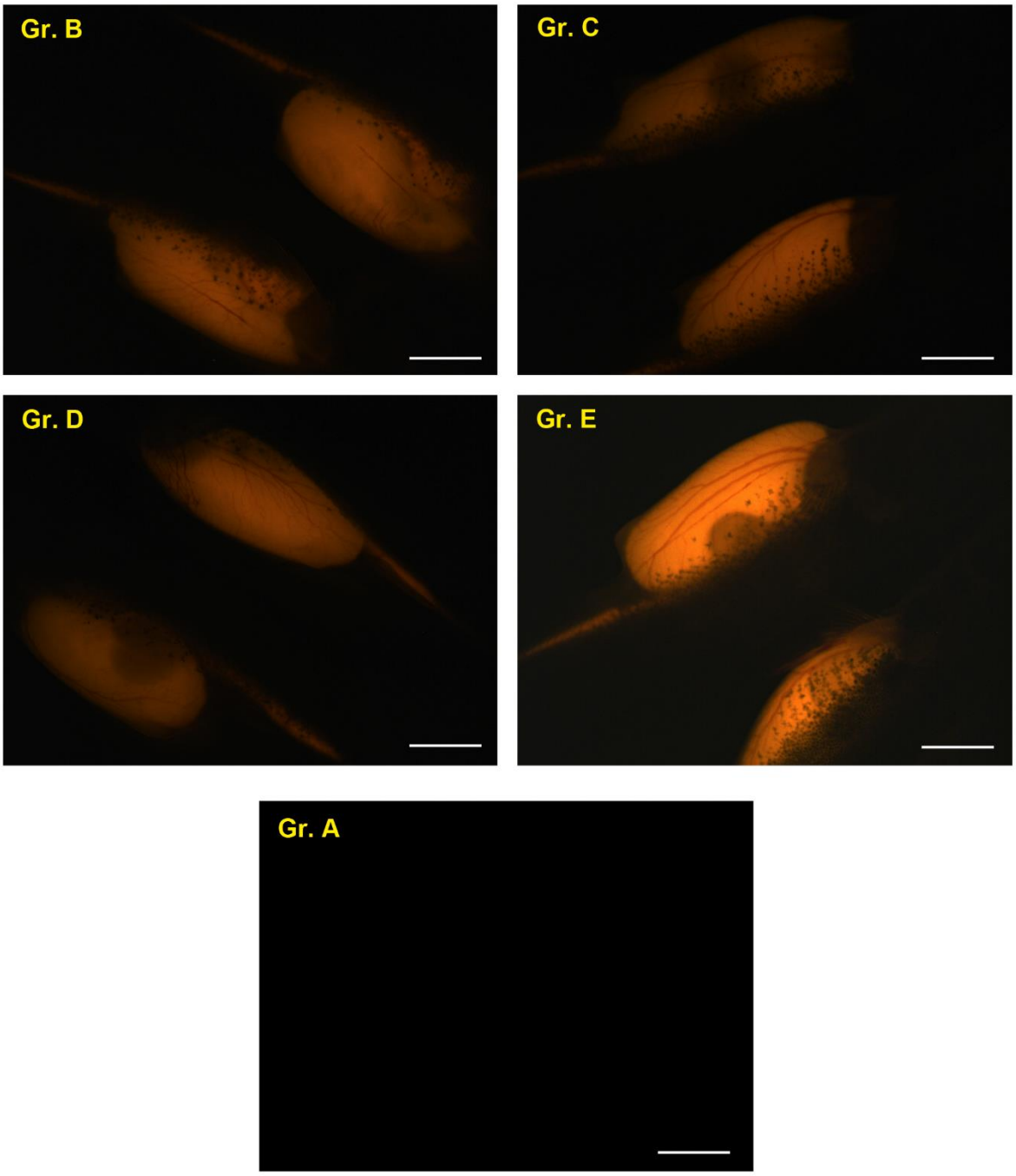

Figure 5

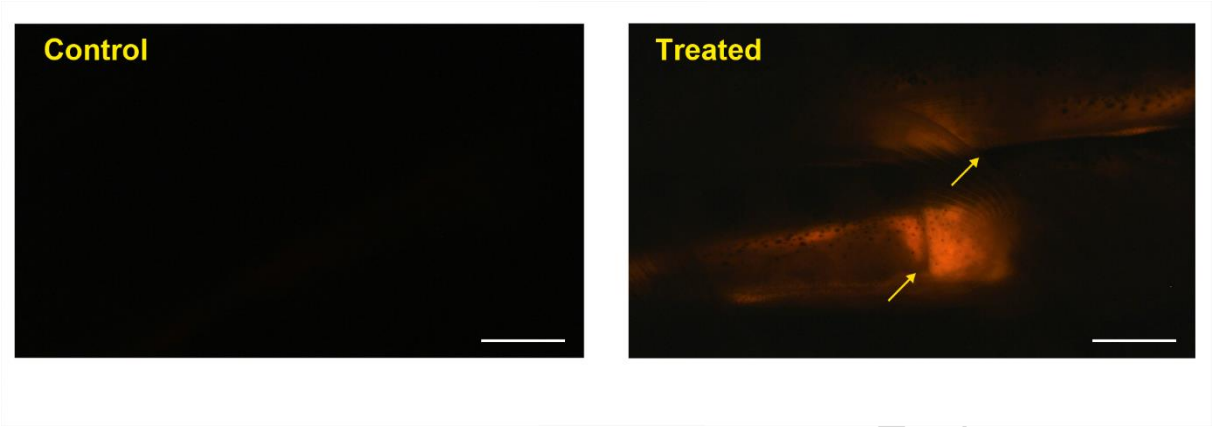

Figure 6