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Tutorial

Ultrastructural morphology of the envelope of Dover sole *Solea solea* eggs from fertilization until hatching with emphasis on sample preparation

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ABSTRACT

This study is the first to describe the ultrastructural morphology of the envelope of *Solea solea* eggs from fertilisation until hatching. Defining the ultrastructural morphology of fish eggs is important for species identification and may assist in predicting the effect of external influences on these early life stages. In first instance, various fixation and embedding protocols were assessed to explore the morphology of the egg envelope, whereby the encountered difficulties were highlighted. The successful protocol for SEM proved to be combined fixation with 4% glutaraldehyde in 0.1 M cacodylate buffer for minimum 4 h with post-fixation of 2 h with 1% OsO_4 in 0.1 M cacodylate buffer. For TEM, puncturing the egg envelope during the first steps of the fixation protocol was necessary to allow the embedding medium to penetrate through the egg envelope. Based on both scanning and transmission electron microscopical examination, three distinct layers were discerned in the egg envelope. During the development of the fish embryo, a change in the outer structure of the egg was observed. Scanning electron microscopical examination of one day post-fertilisation eggs (DPF) revealed a homogeneous outer layer, displaying a large number of pores uniformly distributed on the surface of the egg envelope. Starting from 2 DPF parts of the outermost layer or two outer layers peeled off. The second deeper layer showed larger pores, with less defined edges. In the third innermost layer irregular indentations were noted. On transmission electron microscopy the first outermost layer of 1 DPF eggs clearly folded into the pores. The second layer was more electron dense, had a uniform appearance and did not cover the surface of the pores. The third innermost layer was much thicker and possessed indentations. A total number of 12 undulating zones were discriminated based on different degrees of electron density. Prior to hatching, the compact structure of the innermost layer was distorted by dispersed holes and tears.

1. Introduction

To meet the rising demand for fish induced by the continuously growing human population, aquaculture has expanded very rapidly and is now the fastest growing food producing industry in the world. Fish and fishery products represent a widespread, affordable and healthy source of protein. With stagnating global capture fishery production, aquaculture has the potential to produce higher amounts of safe and quality fish in the future, to meet the increasing demand for (sea)food (FAO, 2014). The growing importance of the aquaculture sector during recent decades combined with indications of market saturation for some established species like sea bass (*Dicentrarchus labrax* L.), render a diversification of fish species and products imperative (FAO, 2014). Flatfish species constitute important and credible new aquaculture candidates as exemplified by Dover sole *Solea solea* (Linnaeus 1758) exhibiting high flesh quality and important market value, and being the subject of an increased consumer demand (Imsland et al., 2003). As for other cultured

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marine teleost species, production has been severely hampered by difficulties in larval rearing as manifested by low and unpredictable survival rates of both eggs and larvae (Boglione et al., 2013a; Boglione et al., 2013b). *Solea solea* is considered to have a moderate vulnerability to (local) extinction caused by fisheries (Cheung et al., 2005) and produces pelagic eggs, which hatch around 1400° h (at 13 °C) after fertilisation (Devauchelle et al., 1987). Despite its growing significance, no information is available on the egg structure and composition, which could assist in further understanding the development of *S. solea* and improve its chances for obtaining the status of a successful aquaculture species.

Overfishing and destruction of marine habitats especially affects benthic fish populations as indicated by landings for plaice (*Pleuronectes platessa* Linnaeus 1758) that declined more than 97% (Thurstan et al., 2010). Today, electrofishing is a promising alternative fishing technique for beam trawling when targeting flatfish and shrimp to mitigate at least the destruction of benthic habitats. The possible effects on marine organisms have been extensively studied during recent years (Soetaert et al., 2013) with the exception of the impact of exposing early developmental stages, including eggs, to electrical pulses. Such data are crucial, as electrofishing may occur over these shallow active spawning areas of many species, including *S. solea*. Earlier studies on fresh water species have indicated a reduced survival of embryos if exposure to the electric field happened during the first embryonic stages, in particular around early cleavage and epiboly (Godfrey 1957; Dwyer et al., 1993; Muth and Ruppert, 1997; Henry and Grizzle, 2004; Bohl et al., 2010). Bohl et al. (2010) suggested that the effect on survival was not related to the species differences in themselves, but to physical characteristics (i.e. size) of the eggs rendering a prediction of the vulnerability in untested species possible based upon morphological data.

The teleost egg is surrounded by a complex multi-layered envelope, composed of protein fibres in a protein matrix (Iconomidou et al., 2000) with a specific (ultra)structure and chemical composition. In addition, the structure and thickness of the egg envelope also varies depending on the developmental stage of the egg or the ecological conditions of the parental population (Chen et al., 1999). The egg envelope undergoes structural and biochemical changes during oocyte maturation (Ravaglia and Maggese, 2003; Fausto et al., 2004; Bian et al., 2010). Furthermore, immediately after fertilisation large changes in egg morphology occur to prevent polyspermy and to protect the developing embryo (Iwamatsu and Ohta, 1981; Kobayashi and Yamamoto, 1981; Iwamatsu et al., 1991; Iwamatsu et al., 1993; Mekkawy and Osman, 2006). The most important proteins and glycoproteins of the egg envelope have been characterized in different species (Scapigliati et al., 1994, 1995; Oppen-Berntsen et al., 1990; Baldacci et al., 2001). More recently, changes in these proteins are linked to environmental pollutants, making the egg envelope a potential sensitive biomarker (Arukwe et al., 1997; Arukwe and Goksoyr, 2003; Berois et al., 2011). In addition, the ultrastructural morphology of both the outer surface of the egg envelope and of the micropyle are frequently used for species identification and phylogenetic classification (Riehl, 1980; Lonning et al., 1988; Chen et al., 1999; Li et al., 2000; Gwo, 2008), again reinforcing the importance of the availability of these morphological data.

Considering the above, the aim of this study is to investigate the overall ultrastructural morphology of the egg envelope of *S. solea* embryos during development.

There is considerable variation in the nomenclature used to describe the external layers of teleost eggs, hampering comparison between studies. Frequently used terms are zona radiata (Breining and Britz, 2000), chorion (Lonning and Davenport, 1980; Chen et al., 2007), egg membrane (Stehr and Hawkes, 1979), vitelline envelope (Ravaglia and Maggese, 2003), egg envelope (Scapigliati et al., 1994) and egg shell (Baldacci et al., 2001). In this study, the term egg envelope was used when referring to the outermost layers of the fertilised fish egg, surrounding the fish embryo. To describe the openings in the outer layers of the egg envelope, the term pore was employed which coincides with most of the literature relevant in this domain.

2. Material and methods

2.1. Sample collection

Solea solea embryos were obtained from the Institute for Marine Resources & Ecosystem Studies (IMARES) in IJmuiden, the Netherlands. Eggs were naturally spawned, fertilised overnight and collected the next morning. Following transportation in natural seawater with a salinity of 32, embryos were acclimatized with and further incubated in aerated artificial seawater (Instant Ocean Aquarium Systems, Mentor, Ohio; salinity 34; 9 h light/15 h darkness). Embryos were determined as developing when the eggs remained floating (dead eggs sank to the bottom of the tank when aeration was discontinued) and no white discoloration was observed. Only developing embryos were sampled. For scanning electron microscopy, embryos were incubated at 17 ± 1 °C and sampled 1 day post-fertilisation (DPF), 2DPF and 3DPF. Hatching followed during the night between 3DPF and 4DPF. For transmission electron microscopical examination, embryos were incubated at 15 ± 1 °C and sampled on 1DPF, 2DPF, 3DPF and 4DPF. Hatching occurred during the following night.

2.2. Scanning electron microscopy

Various fixation protocols in terms of fixative constituents (single and in combination) and concentration and fixation times were tested on minimal 20 embryos 1DPF (Tables 1 and A1). After fixation, the embryos were washed three times with cacodylate buffer (0.1 M). Post-fixation with 2% osmium tetroxide (Os_2O_4) followed by washing three times with cacodylate buffer (0.1 M) was an optional further step. Thereafter, dehydration of the embryos was performed in a graded series of ethanol (10–95% in water, Table A1), followed by a graded series of acetone (10–100% in ethanol, Table A1). Finally, samples were placed in 100% acetone and incubated at room temperature overnight. The next day, the embryos were dried with a Balzers CPD 030 critical-point dryer (Leica, Diegem, Belgium) and platinum-coated using a JEOL JFC-1300 Auto Fine Coater (Jeol Ltd, Zaventem, Belgium). The specimens were observed and photographed with a JEOL JSM 5600 LV scanning electron microscope (Jeol Ltd) under an accelerating voltage of 15 kV.

Stereomicroscopic examination of the embryos was performed after fixation and both following dehydration with ethanol and acetone. Only those protocols resulting in at least 75% of the embryos being fully round were further employed (Table 1). The protocols that, having gone through all steps, yielded round, intact embryos for SEM were repeated twice in time, using different embryo batches of the same age.

To determine the size of the embryos, 24 fertilized embryos of each sampling day were examined based on the SEM images. For each embryo, the best fitting circle was drawn and its diameter measured with Scandium 5.2 software (Soft Imaging System, Olympus N.V.). This method was also used to establish the micropyle diameter. The difference in embryo diameter between the three sampling days was tested using one-way ANOVA, post hoc comparisons were performed using a Tukey HSD test. Statistical results were considered significant when p-values < 0.01. The density of pores was determined for 26 intact embryos of 1DPF, based on SEM images. A detailed image of the surface of the egg envelope