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Research Article

Fluorescent Methods for Test-Analysis of Plankton Fish Eggs

Victoria V Roshchina[1]*, Valerii A Yashin[1], Tatyana N Petrova[2] and Vladimir I Maltsev[2]

[1]Institute of Cell Biophysics, Federal Research Centre, Pushchino Scientific Centre for Biological Research, Russian Academy of Sciences, Pushchino, Moscow Region, Russia

[2]TI Vyazemsky Karadag Biological Station, Institute of Southern Seas of the Russian Academy of Sciences, Kurortny, Feodosiya, Cream, Russia

***Corresponding Author:** VV Roshchina, Institute of Cell Biophysics, Federal Research Centre, Pushchino Scientific Centre for Biological Research, Russian Academy of Sciences, Pushchino, Moscow Region, Russia.

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Abstract

Luminescence microscopy with microspectrofluorimetry have been applied to the study of the fluorescence of fish egg in the ichthyoplankton to identify the state - development or damage. The autofluorescence and fluorescence after histochemical treatment for the biogenic amines (dopamine, histamine and serotonin) as stress indicators of fish eggs from the species of the Black Sea - *Engraulis encrasicholus L., Mullus barbatus ponticus* Es., *Trachinus draco* L., *Trachurus mediterraneus ponticus* Aleev have been studied. The application of the fluorescent methods may be used for testing of fertilized or damaged eggs at the early stages of development.

Keywords: *Biogenic Amines; Dopamine; Embryo; Fish Egg; Histamine; Luminescence Microscopy; Microspectrofluorimetry; Serotonin; Shell; Yolk*

Introduction

Egg (approximately 0.1 - 5 mm in diameter) is of the step in the developing of the mature fish. Moreover, eggs themselves is a useful product in human nutrition. The state of planktonic fish eggs needs a control by various methods in order to know their fertilization and quality of the living material in ecological monitoring and fishery.

Animal eggs structures develop from a formation of oocytes (oocyte is a developing female structure that cannot yet bind sperm or be fertilized) to mature female cells [1,2]. This mature egg consists of nuclear part in cytoplasm (pronucleus) covered with yolk, and this all is surrounded with the yolk membrane. Outside the egg membrane, there is a shell with several layers. Recently the attention has been arisen to the fluorescence of plankton eggs in visible spectral region [3] as possible test for living and dead samples by luminescence microscopy with microspectrofluorimetry [4,5]. In this paper, we reflected the process of the development and damage determining on the examples of the four fish species of Black Sea.

Purpose of the Study

The purpose of the study to apply fluorescent methods to testing of the damage and development of fish eggs that it can be used in practice of fishery and nutrition.

Materials and Methods

Objects: The objects of research were samples of fish eggs from plankton in the Black Sea basing near Karadag Biological Station of Feodosia, which are (Table 1) valuable commercial species [6-8].

Fish species	Trivial name	Family
Engraulis encrasicholus L.	European anchovy	Engraulidae
Mullus barbatus ponticus Es.	Red mullet or Barabula black sea or mullet lamb roe	Mullidae
Trachinus draco L.	Greater weever	Trachinidae
Trachurus mediterraneus ponticus Aleev.	Black Sea horse-mackerel	Carangidae

Table 1: *Eggs from fish species studied.*

Living or dead eggs' samples were collected at spawning and fixed with 4% or 10% formalin.

Measurement of fluorescence

Autofluorescence: Autofluorescence of samples in formalin was observed and photographed directly on slides using a Leica DM 6000 B luminescent microscope (Germany) and the MSF-15 microspectrophotometer/fluorimeter [9,10] with the Levenhuk M300 Base camera (USA). The fluorescence spectra were recorded using the MSF-15 microspectrofluorimeter (LOMO, Russia). Fluorescing cells were analyzed and photographed.

Histochemical methods for determination of biogenic amines: Fluorescent histochemical determination of biogenic amines (dopamine, histamine and serotonin) within cells, was carried out according to the methods primary described for animal cells [11] and applied for plant cell as well [9,10]. Microspores were put on object glasses (slides) and moistened by drops of 1% aqueous solutions of glyoxylic acid for dopamine, or o-phthalic aldehyde for histamine or formaldehyde for serotonin. After 10 - 20 minutes of staining with the reagent, samples were dried at 50 - 80°C during 5 - 10 minutes.

Fluorescence reactions of forming products was studied under luminescence microscope Leica DM 6000 B or by camera Levenhook (USA) at the excitation by light 360-380 nm. The fluorescence spectra recorded by microspectrofluorimeter MSF-15 (LOMO, Sankt-Petersburg). Histochemical reactions repeated (up to 3-5 times).

Statistical analysis

Results of the fluorescence intensity at 460 nm were expressed statistically with a standard error of mean +SEM and has been shown graphically on figures (n = 4 - 5 subject glasses with 3 cells).

Results and their Discussion

Autofluorescence of living and dead eggs: Analysis of planktonic fish eggs has not yet included a testing of their autofluorescence in visible spectral region. Meanwhile, the position of the maxima of the fluorescent components and the intensity of egg emission in visible spectral range potentially could be useful for testing its condition, development and fertilization in modern environmental practice.

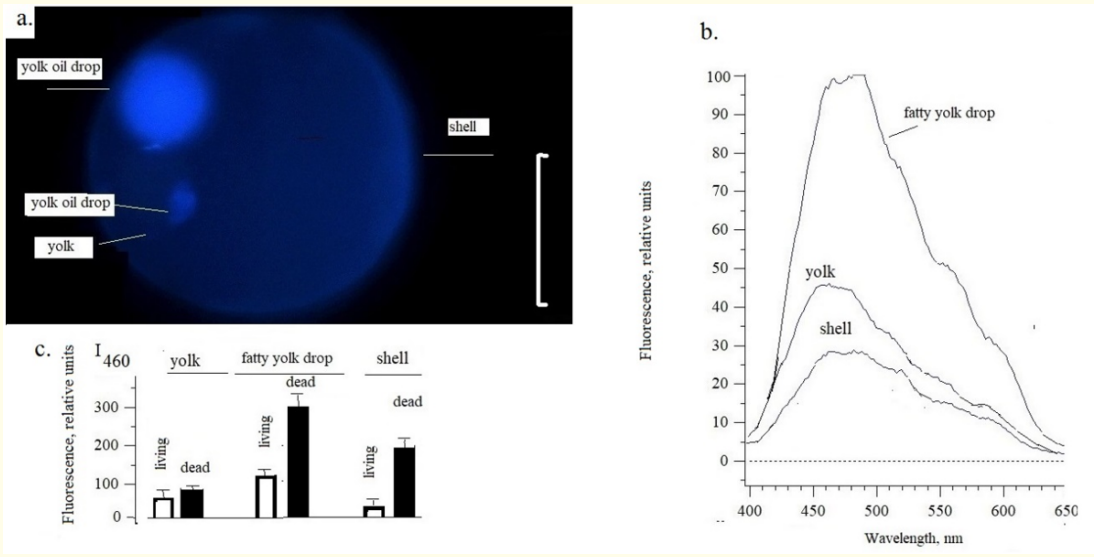

Figure 1: *View of red mullet egg under Leica DM 6000 B luminescence microscope (a) and the fluorescence spectra (b) when excited by ultraviolet light 360 - 380 nm. (c) Fluorescence intensity at 460 nm in different parts of living and dead cells. Optical probe 5 µm. Bar = 150 µm.*

Our primary experiments with fish eggs have begun from autofluorescence of red mullet fish and horse-mackerel eggs because their cellular probes were more successful in the analysis [3]. For the species it has been demonstrated bright and intensive lightening. Here we compare the data with European anchovy and great weever and confirm main of them for new species too. We saw fluorescence of red mullet unfertilized egg in blue or green after excitation by ultra-violet or violet light (Figure 1 should be as most suitable example) that was also observed in all studied species, and their spectra of emission were measured and have maxima in the same regions, both in blue and green. Using optical probe of microspectrofluorimeter, sizes from 0.5 mm up to 2 µm [3] we saw the emission of some parts of egg - yolk, yolk fatty drop and shell. As seen on figure 1a, most bright and intensive its lightening was peculiar for yolk and especially for yolk fatty drop within the cell. Using optical probe of microspectrofluorimeter, sizes from 0.5 mm up to 2 µm [4,5] we saw the emission of some parts of egg - yolk, yolk fatty drop and shell. More intensive was a fluorescence of yolk fatty drop under various used lights for excitation- ultraviolet 360 - 380 nm (Figure 1b), blue 430 - 450 nm and green 480 - 500 light with maxima 460 - 470 nm, 520 - 550 nm and 560 nm, relatively. The emission in blue associated with NAD/NADH and in green - with Flavin's (riboflavin) of mitochondria [12]. Some pterins also emitted in blue under ultraviolet light [13,14]. Fluorescence of carotenoids is possible at 530-540 nm [15]. The most intense emission with a maximum of 540 - 550 nm in the green region was noted for the cell after excitation by violet light [3].

The comparison of blue emission at 460 nm in living and dead eggs of red mullet gives a possibility of the selection. Dead cells have most intensive emission (Figure 1c). Moreover, highest enhancement was well seen for yolk fatty drop and shell. When yolk, yolk fatty drop and shell was excited by green-yellow light 515 - 550 nm, there is no visible fluorescence in orange-red if the egg was unfertilized. Four species demonstrated similar results. Unlike red mullet, eggs of other fishes were lack of fatty yolk drop or had small one that has most intensive emission, their lightening in blue and green was evenly distributed in yolk.

Main difference was for the red region of the spectrum after contact with spermatozoid [3]. Red emission in yolk fatty drop that marked with small maximum 630 - 650 nm appeared as seen on figure 2a for red mullet. Main red intensive fluorescence belongs to yolk in every species studied, but especially in yolk fatty drop of red mullet. Photographs done under a luminescent microscope show the natural color of fluorescence.

Red autofluorescence in fertilized eggs: Red fluorescence was absent in unfertilized samples, and appeared in fertilized eggs just after the spermatozoid (premium) contacted with female cell [3] and proposed may be a marker of the process. Unlike unfertilized egg, figure 2 shows first changes in fluorescence just or after the contact with spermium (spermatozoid). According Gilbert [1], fertilization involves the contact and fusion of the mature sex cells, the sperm and egg named as gametes. Clearest picture is well seen for big yolk fatty drop. It should mark that great new was the appearance of red fluorescence of yolk fatty drop (Figure 2a). Clear maximum 655-660 nm in yolk was seen in the emission spectra in four our samples of developing eggs. If to retrace the latest stage red fluorescence seen in yolk, when animal pole with non-fluorescent blastomeres was appeared (Figure 2b). The red emission seen in embryo forming in egg too. Moreover, this phenomenon is peculiar to all analyzed species. Earlier we demonstrated the absence of red fluorescence in living and dead cells of red mullet and horse-mackerel [3].

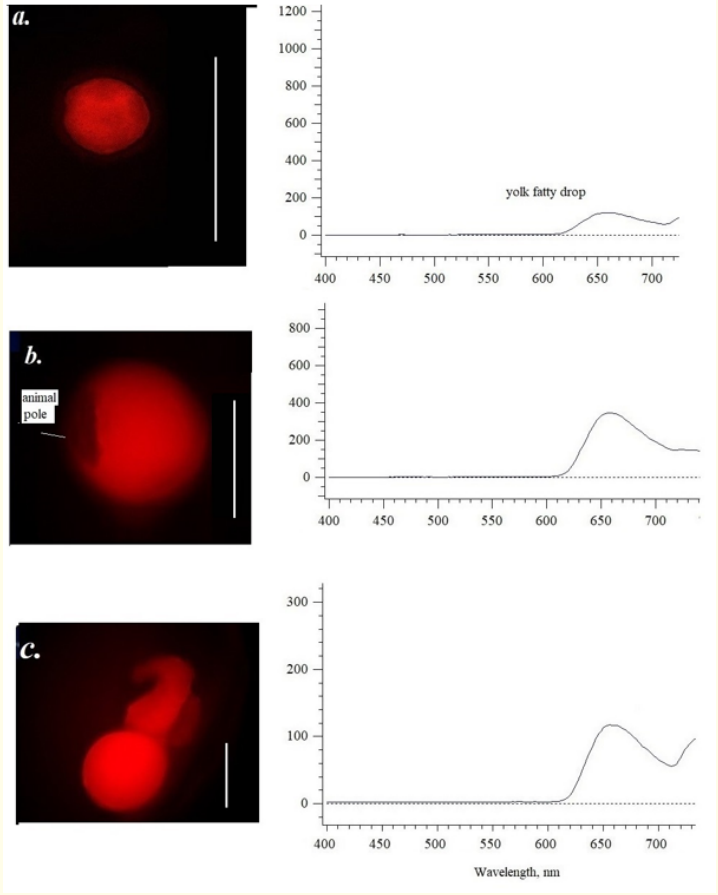

Figure 2: *The fluorescence spectra of the red mullet egg (fixed in 4% formalin) when excited by light 515-550 nm on the different stages of the development: a - after contact with spermatozoid, bar = 300 µm; b - stage of the animal pole formation (here non-fluorescent blastomeres are), bar = 300 µm; c - embryo formation, bar = 150 µm. Registration with a microspectrofluorimeter. Optical probe 5 µm.*

According to the monograph of Mikulin [13], fish yolk may include pigments, not only lipophilic compounds such as carotenoids- antioxidants of lipid peroxidation, but also -proteins (porphyrin-containing structures such as cytochrome b_{560} and other heme-proteins). We only hypothesized about the inclusion in the visible red fluorescence some carotenoids and iron-containing heme-proteins. There is the information that yolk of fish eggs contains heme-proteins [13]. Some porphyrins excited by ultraviolet 400 nm, emitted with maxima in region 600-750 nm - 630, 660 and 690 nm [15]. Hematoporphyrin from blood erythrocytes (hemoglobin) treated with 0.5-1.5 N HCL or sulfur acid fluoresces with maxima 580- 588, 600, 625, 660, 625nm [12], According to some authors [16-21] acetone extracts of blood elements have maxima 585 and 630 nm in the fluorescence spectra at the excitation 400 nm. Therefore, we can consider that visible red emission relates to one and same components - porphyrins (likely as precursors of blood hemoglobin as observed only in living samples). Red autofluorescence may serves as an indicator of fertilized and developing eggs.

Histochemical identification of developing and damaged eggs: Biogenic amines dopamine, histamine and serotonin are known as neuromediators in animal cells, although they are found in plant and microbial cells [22]. They regulates main functions in mammalians and in high concentrations are supposed as stress compounds. We tried to find the compounds in fish eggs. As seen on figure 4, biogenic amines are met in various parts of the egg cell in analyzed fish species. In the analysis, we differed small amounts of dopamine and histamine in undamaged cells in yolk, yolk fatty drop and shell of red mullet, while in other species the concentration of histamine is high both in yolk and yolk fatty drop. Earliest phase of development or unfertilized state demonstrated lack of serotonin et all in most samples, however, this amine is clearly marked in European anchovy and in lesser degree - in Greater weever in yolk. We also studied the fluorescence of blastomeres and visible larva and found in 10 times more higher fluorescence related to dopamine and histamine in developing eggs of red mullet, lesser - in horse-mackerel and European anchovy. Besides, larva eggs of the last species contained high amount of serotonin. Serotonin was observed also in yolk of European anchovy and Greater weever. As a whole, we supposed that histamine is more real indicator of the egg damage that is very important for analysis of its nutrition quality. Basing on earlier stage of development, one could conclude the occurrence of small amounts of all analyzed amines. Although the amines in small amounts are known as normal for any animal organism, the compounds in high concentration is a stress reaction, including a damage. Among studied species, only in European anchovy and Greater weever we saw the serotonin presence. In any cases, the problem needs new following studies.

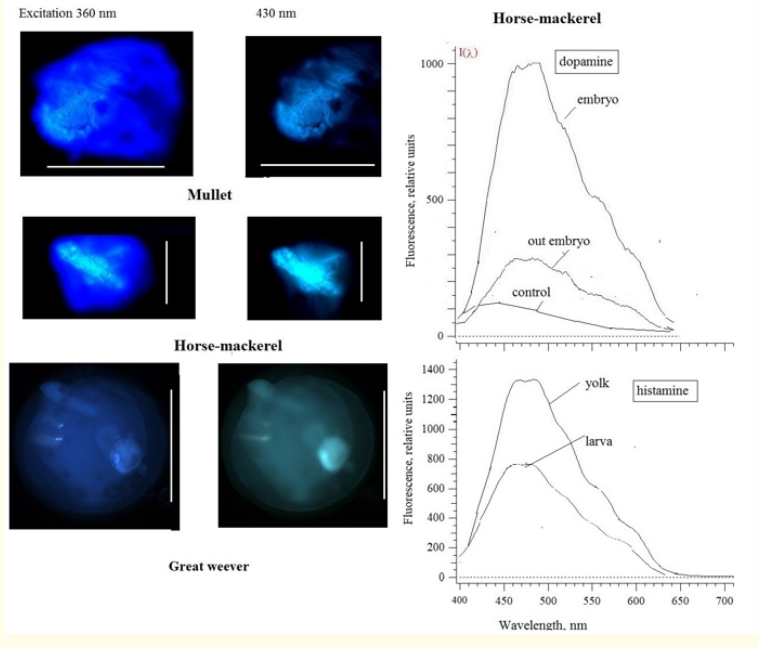

Figure 3: *Examples of fluorescent images under Leica DM 6000 B luminescence microscope (left) and the fluorescence spectra (right) stained with reagents for biogenic amines and recorded by microspectrofluorimeter MCF-15. Excitation by ultraviolet light 360 - 380 or 430 nm (left images) and 360 - 380 nm (right spectra). Optical probe 5 µm. Bar = 300 µm.*

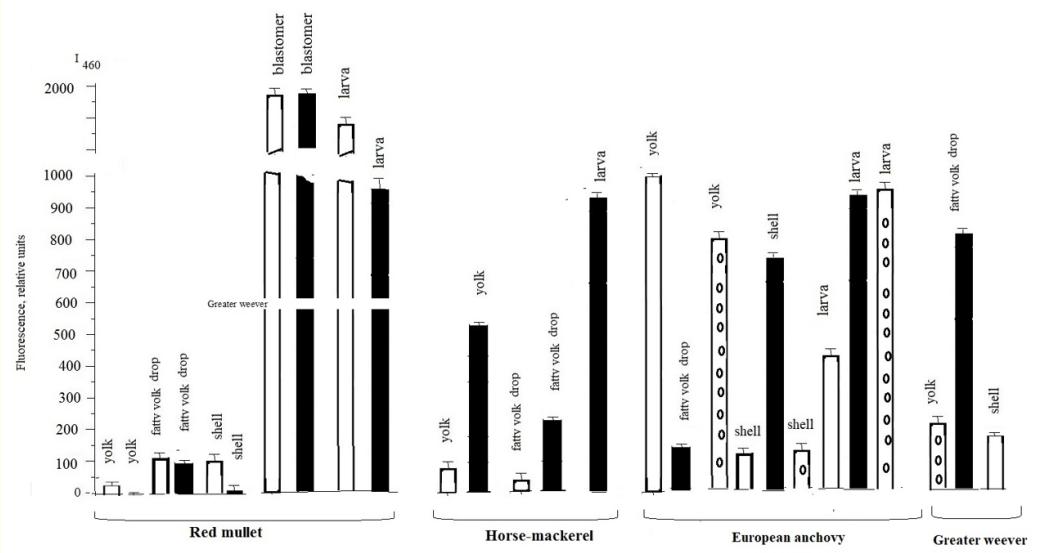

Figure 4: *The fluorescence intensity after the histochemical reactions of fish eggs for dopamine (white), histamine (black) and serotonin (with rings) when excited by ultraviolet light 360 - 380 nm.*

Conclusion

First investigations of the fish egg autofluorescence and histochemical fluorescent reactions on biogenic amines show the possibilities to use the luminescence method in practice of fishery and nutrition. Red autofluorescence may be related mainly to porphyrins that have seen at earlier stage of the egg development just after the fertilization. This phenomenon may be considered as an indicator of fertilization and development. Histochemical fluorescent method with special reagents for biogenic amines has been recommended in the indication of the cell damage basing on the intensity of blue emission at 460 - 470 nm.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Victoria V. Roshchina, the author of main conception of the work, receiver of all experimental data, and she has written the paper. Valeri A. Yashin, leading specialist in optical methods, Tatyana N. Petrova and Vladimir I. Maltsev - collection of fish eggs and their identification.

Data Availability

The datasets generated during and/or analysed during the current study are available in the [Name] repository [Persistent link to datasets].

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