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Non-Invasive Evaluation of Egg Quality and Early Life Stage Development of Medaka Fish (*Oryzias latipes*) by Means of Morphological Image Analysis Methods

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Introduction

Animal models are often used to investigate environmental contamination by various industrial wastes and the toxicity of various potential drugs and substances used in the production of clothes and food. The tendency nowadays is to reduce the cost of these experiments and the number of organisms, in concern for animal welfare. A notable example is the awareness of the ecotoxicologist community of the pharmaceutical industry to apply the principles of replacement, reduction, and refinement (the 3Rs). This was established by Russell and Burch (1959), in the context of regulatory environmental assessments [1]. One of the alternatives proposed is to use the early life stages of fish as an experimental model [2,3]. Fish embryos and larvae are generally considered to be the most sensitive stages in the life cycle of teleosts [4].

Investigation of early life stages is also important for estimating the chances of survival of particular fauna species facing global environmental changes.

Amongst the most popular species used for such investigations are zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), rainbow trout (*Onchorhynchus mykiss*) and fathead minnow (*Pimephales promelas*). These popular species are mostly selected because of their developmental and physical features. For instance, adult zebrafish can produce a large number of translucent embryos every week, by external fertilization, and the embryogenesis is well characterized [5,6,7]. Medaka (*Oryzias latipes*) have similar features [8]. Visual investigation of embryo development is totally non-invasive and gives promising results. Recent technologies allow visual investigation not only of early life stages but also of adult animals. A product of advanced genetic techniques is the so called "see-through" medaka [9], which is genetically deficient of pigments and whose internal organs can be observed. Digital imaging could be used in such investigations for quantitative evaluation and more reliable results.

Several standards (OECD 210, ASTEM E 1241-98, OECD 212, ISO 12890, DIN 38415-T6) are regulating the toxicological characteristics and endpoints used for evaluation. The toxicological endpoints are cumulative mortality, pro-larval hatching (rate, time for 50%), larva pro-larval length and weight, larval abnormalities (morphology, behavior), coagulated eggs, somite development, tail detachment and heartbeat.

Evaluation of oocyte quality is one of the methods used not only in environmental tests but in infertility treatment too. Many important events are triggered in the oocyte at fertilization. One of these events – the cortical reaction – is a secretory event involving a wave of exocytosis. It helps prevent polyspermy and leads to hardening of the envelope (chorion) surrounding the oocyte which is equivalent to the *zona pellucida* in human eggs. This reaction could be observed as changes in the distribution and density of cortical granules. It is possible to evoke this reaction artificially, triggering activation of the oocyte by an electrical stimulus. This evokes all the events normally triggered by fertilization apart from subsequent cell division for which the sperm centriole would be required. Quantitative evaluation of density, number of cortical granules and the dynamics of changes provide new possibilities for studying secretion and egg quality. The quantitative evaluation of the number and distribution of cortical granules by fluorescent imaging was reported in [10]. However, invasive staining procedures were applied to make the cortical granules

visible for the imaging system. The procedures make the method unsuitable for most environmental, toxicological or clinical investigations. The application of advanced methods of digital image processing could allow us to achieve similar results and make the evaluation of quality of eggs possible in the desired environment in a non-invasive way.

Disorders in the formation of the cardiovascular system of zebrafish embryos are reported as an indicator of environmental contamination by cadmium [4]. Fluorescein labelled carboxylated latex micro-beads were used to visualize the cardiovascular system of the embryo. Visualization of the cardiovascular system is very complicated and invasive for the embryo. Special image processing methods could permit visualization of the cardiovascular system in a non-invasive way.

In general, characteristics and endpoints (as in the examples provided here) are determined by investigatory technologies that are already available. Advanced methods for digital imaging in many cases could allow non-invasive investigations in desired environment and also allow more reliable quantitative evaluation of the parameters.

In this study we demonstrate how advanced image processing methods could be applied in the evaluation of egg quality and investigation of the development of embryos of the fresh water teleost fish, medaka (*Oryzias latipes)*. Egg quality (evoked cortical reaction) and two parameters characterizing development of the cardiovascular system (vascularization of the embryo and detailed analysis of its heart rhythm) were also evaluated.

Methods

Intact unfertilized (nonactivated) oocytes were obtained following natural oviposition from medaka fish (O*ryzias latipes*) raised in sea water collected from Cancale, North Brittany (France). Such oocytes were osmotically shrunk and for successful activation had to be reswollen in 10% or 25% sea water diluted with distilled water. Oocytes were activated electrically with a +/- 10 volt pulse of 1 to 10 milliseconds duration through Ag/AgCl electrodes placed on either side of the oocyte on a glass slide.

Bright field images of developing embryos of medaka (O*ryzias latipes*) placed in tap water were taken every day starting from the 2nd to the 8th day postfertilization.

Images were taken using a Zeiss Axiovert 35 inverted microscope equipped with a Moticam 3000 (Motic, China) digital camera connected to the personal computer with a 1.5 GHz Intel Centrino processor and 512 MB RAM. Images were taken at 760x520 pixels resolution and 24-bit color encoding. For the egg cortical reaction frames were taken every second for two minutes starting from the beginning of electrical stimulation. Development of the embryo was recorded every 15 seconds at 15 frames/second and 320x240 pixels resolution. All image processing calculations were made with software elaborated using the MATLAB® Image processing toolbox.

Evaluation of the area of the egg covered by cortical granules: Cortical granules are spherical objects of a certain size on the image. Oil droplets are also spherical objects but of varying and often much bigger size. Mathematical morphology methods based on Minkovski addition and subtraction were applied to determine size [11]. Granulometry likens image objects to stones whose sizes can be determined by sifting them through screens of increasing "mesh" size and collecting what remains after each pass. Image objects are sifted by opening the image with a structuring element of increasing size and counting the remaining intensity surface area (summation of pixel values in the image) after each opening. The opening procedure is a combination of erosion of the original image A by structuring element B followed by dilation of the resulting image by the same structuring element B:

$$A \circ B = (A \Theta B) \oplus B. \qquad (1)$$

Preliminary granulometry measurements of images using "disk" structuring elements revealed a radius of cortical granules of 5-6 pixels on the registered images. All round objects with a radius of 5-6 pixels were extracted subtracting images obtained after opening the original image with a structural element of radius 7 from images obtained after opening with a structural element of radius 5. More detailed information about fast morphological image transforms using bitmapped images is presented in [12]. Images containing extracted round objects were converted into black and white format, assigning a value of "1" or "0" according to the condition:

$$a_{j,k} = \begin{cases} 1, & x_{j,k} \geq 0.3 \cdot \max(X), \\ 0, & x_{j,k} < 0.1 \cdot \max(X), \end{cases} \qquad (2)$$

where $\boldsymbol{X}$ – the array of pixels of the picture. Coefficient values of 0.3 and 0.1 were selected during preliminary tests of the method. So, values of pixels in the transformed image belonging to the recognized objects were equal to "1" and zeroes were assigned to the rest of the pixels. The area covered by objects on the ordinary frame S_i was estimated as the sum of pixel values:

$$S_i = \sum_{j=1}^{m} \sum_{k=1}^{n} a_{j,k}, \qquad (3)$$

where $\boldsymbol{a}$ – values of the pixels, m and n – dimensions of the frame – 760 and 520 respectively.

Visualization of blood vessels: Moving blood cells are visible in the vessels of the developing embryo and differences between the values of pixels of consequent frames in the movie can trace them. The accumulated difference of images was calculated as follows:

$$\hat{A} = \sum_{i=1}^{N} A_i, \qquad (4)$$

where $\boldsymbol{A_i}$ – a difference of subsequent frames:

$$A_i = \left| F_i - F_{i-1} \right|. \qquad (5)$$

$\boldsymbol{F_i}$ and $\boldsymbol{F_{i-1}}$ are the arrays of pixel values of subsequent frames. Elements of matrix $\hat{A}$ were filtered using the threshold level to get rid of some artifacts and non significant image distortions and converted into black and white format. Values of "1" or "0" were assigned to the elements of the matrix according to the same condition (2) used for cortical granules. The threshold values were also selected during preliminary tests of the method. Accumulations of white pixels ($a_{j,k}$=1) were consolidated

into clusters by means of fast morphological image transform using the image closing function:

$$A \bullet B = (A \oplus B) \Theta B, \qquad (6)$$

where ***B*** was a "disk" structuring element of radius 3 [12]. The area *S* covered by these clusters was estimated in the same way as in the case of cortical granules (3). All calculations were made with software elaborated using the MATLAB® Image processing toolbox.

Registration of heart beats: The photo detector (LDR 2-20K Ø4mm) was mounted in the ocular eyepiece of the microscope and together with a DC power source connected to the ADC of the computer's sound card. The microscope aperture was used to hide all the light passing through the embryo except at the location of the beating heart. Illumination changes due to heart movement were converted to an electrical signal containing peaks at the time of heart contraction. The complicated shape of the peaks meant that the amplitude detector could only be used for the preliminary detection of the heart beats. Final adjustment of heart beat moments was performed by maximizing the correlation between the preliminary detected ordinal????? signal peak and the model with a permanently updated shape. The heart beat moments were used to form, so called in cardiology, R-R interval sequences of the heart beats of the embryo. Evaluation of the average heart rate in developing fish embryos is a well known and popular method [13]. However, detailed analysis of heart rate variability could reveal functions of autonomous heart control that should appear together with the developing nerve system. Spectral analysis of fetal heart rate variability is known and used in clinical practice [14]. So we decided to perform spectral analysis of the R-R interval sequences detected from the developing embryos.

Results

Typical pictures of the oocyte before and after activation following electrical stimulation are shown on Fig. 2. One can see the whole area of the oocyte covered by small round objects - cortical granules on the upper left image which was taken before activation. Almost no such objects remain on the bottom left image, which was taken 15 seconds after the start of the activating electrical stimulus. Pictures on the right of Fig. 2 illustrate the results of the image processing procedure. Black and white format images are obtained from the images shown on the left. Only some oil droplets, bigger than the round cortical granules on the initial images were misrecognized as cortical granules, but this does not crucially influence the final result. The calculated area covered by cortical granules before activation was in the range of 30% - 40% of the total area of the oocyte and decreased to 20% - 25% at the end of the reaction. Dynamics of the reaction were different and steady state values after activation were reached in times varying from 15 to 45 sec. The aim of this study was to show that cortical granules could be recognized by this method and the area covered by them could be evaluated. Therefore, we tested the method on as many different oocytes as possible. But in this study we do not provide any comparison of the image processing results in regard to the conditions of oocyte preparation etc. A variety of normalized time courses of area covered by cortical granules following activation of oocytes by electrical stimuli is presented in Fig. 1. Expert control confirmed that recognition of cortical granules in all presented cases was of sufficient quality.

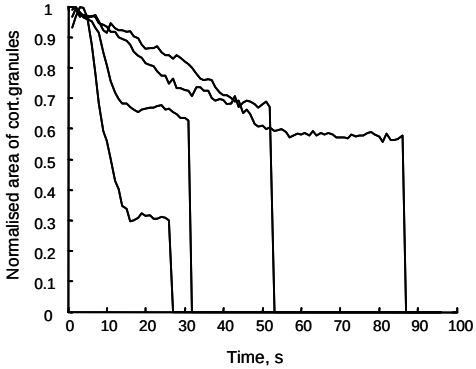

Fig. 1. A variety of normalized time courses of the area covered by cortical granules following activation of oocytes by electrical stimuli.

Typical pictures of medaka embryos on the 2nd and 8th day after fertilization are shown on the left of Fig. 3, upper and bottom images respectively. The image processing result – black and white images with recognized blood vessels marked in white on black background are presented on the right of Fig. 3. The accuracy of marking of the blood vessels was considered satisfactory. The area covered by blood vessels increased in average about 4 fold by the 8th day when compared to the 2nd day after fertilization.

The average heart rate increased during development of the embryo in all registered cases. Time intervals between consecutive heartbeats (R-R intervals) registered in embryos on the 2nd and 8th day after fertilization are shown in Fig. 4. Besides the increase in the heart rate the variability also increases. The power spectrum of the R-R interval sequences is presented in Fig. 5. Some peaks appearing at about 0.6 Hz frequency range on later days of development were present in all registered cases.

Discussion

The recognition of cortical granules of the artificially activated oocyte with sufficiant reliability is a promising result opening a perspective for non-invasive investigations of the quality of oocytes not only in toxicological or environmental investigations, but also in clinical investigations. The limitations of this method are that the area we observe in our pictures is a projection of just part of the sphere concerning both the oocyte and the cortical granules. In fact the observed process reflects exocytosis – release of substances into the perichorionic space of the oocyte - and should be evaluated in terms of volume. Estimation of the total volume of the oocyte would not be a big problem, however, for the estimation of the total amount of substance released from cortical granules we could calculate this only by knowing what part of the total amount the visible granules represent. This would require additional investigations.

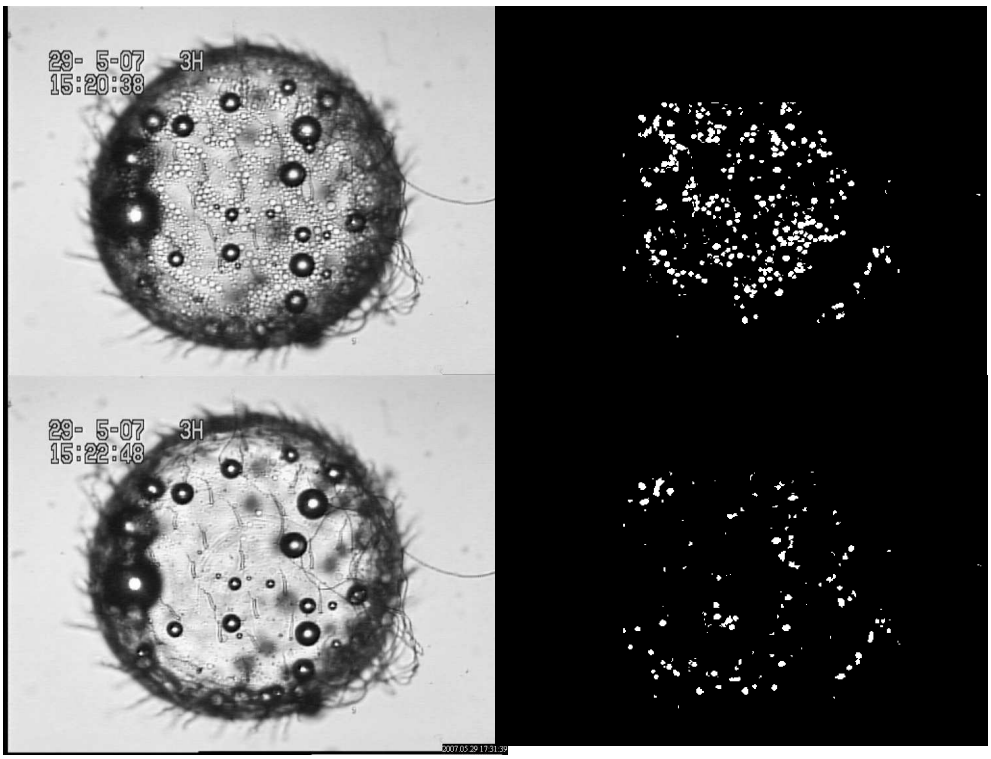

Fig. 2. Typical pictures of an oocyte during electrical activation and the transformed black and white format images of the same oocyte. The upper images show the oocyte at the start of the stimulus and bottom ones after 15 seconds

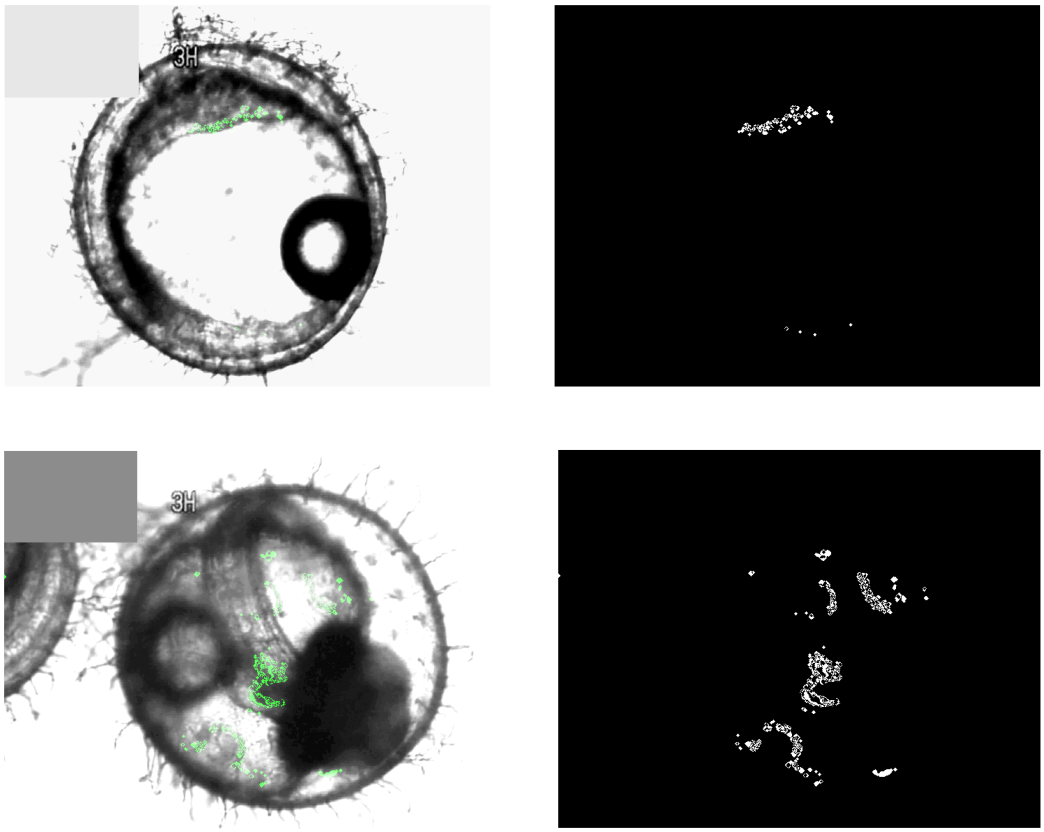

Fig. 3. Typical pictures of medaka embryos and visualized blood vessels in black and white format on the 2nd day (upper images) and the 8th day after fertilization (bottom images). The number of white pixels is 969 in the top image and 4009 in the bottom one

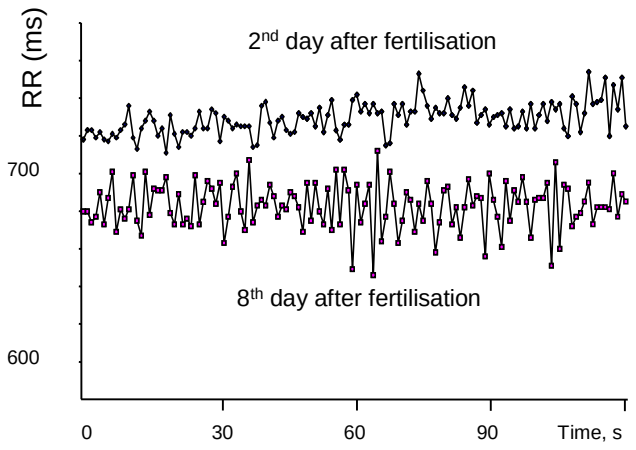

Fig. 4. Time intervals between consecutive heartbeats (RR intervals) in medaka embryos on the 2nd and 8th day after fertilization

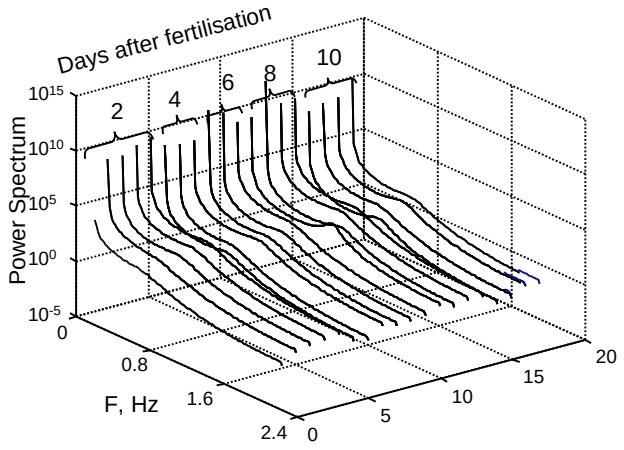

Fig. 5. Power spectrum of R-R interval sequences

Nevertheless, the same part of the oocyte is visible during the whole investigation and our relative figures of dynamics of area covered by cortical granules reflects the oocyte activation process and could allow better evaluation of oocyte quality and the activation process.

Many parameters or key points in embryo development are usually visually evaluated because of the difficulties to elaborate morphological models in a quantitative way that describe the developing structures. In this study we present only the first and easiest to calculate parameter characterizing development of blood vessels. However, we think it is a first step towards application of so called blind image analysis methods successfully used in many areas including biomedical image analysis [15]. It gives generalized quantitative estimates to the objects and structures without detailed morphological analysis. Advanced morphological image analysis could be applied to reveal special morphological development disorders giving them quantitative estimates. We expect that blind image analysis methods could be useful for evaluation of cardiac development disorders in embryos as well as the quality of eggs.

Average heart rate is known as a representative parameter for evaluation of embryo development. Observed heart rate variability in most cases is related to autonomous heart activity control. It reflects nerve system actions regulating heart rhythm according to the demands of the whole organism. Autonomous fetal heart activity control reflecting rhythm variations are known in clinical investigations [14], but there is a lack of information about heart rate variability in fish embryos. Maybe it is due to a lack of suitable methods of evaluation. However, detailed analysis of R-R intervals could allow detection of rhythm disorders – indicators of critical states of the embryo. Time-frequency analysis of R-R interval sequences provides a means of evaluating possible autonomic regulation of heart activity. Appearance of a peak in the 0.6Hz range in the power spectrum of R-R interval sequences in later stages of development in our recordings correlates with widely known frequency characteristics of autonomous heart control. It supports the idea about the possibility of evaluation of heart control mechanisms.

Conclusion

Advanced methods for biomedical imaging and signal processing applied to the analysis of images of early life stages could be a powerful instrument for realizing principles of replacement, reduction, and refinement (the 3 Rs) in pharmaceutical and environmental investigations.

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Early life stage animal models are used to investigate environmental contamination by various industrial wastes and toxicity of various potential drugs or substances used in the production of clothes and food. Estimation of chances of the survival of particular fauna species facing global environmental changes is also done using the same models. This study demonstrates how advanced image processing methods applied for evaluation of egg quality and investigation of the development of the embryos of the fresh water teleost fish, medaka (Oryzias latipes) could extend the possibilities and improve the quality of investigations. Quality of the egg (evoked cortical reaction) and development of the cardiovascular system of the embryo were evaluated using methods of mathematical morphology. Optical registration of the heart beats of the embryos combined with advanced signal processing methods were used for detailed analysis of heart activity. All these methods could be applied to detect and evaluate the developmental disorders of the embryos – indicators of environmental contamination or toxicity of chemical substances. Ill. 5, bibl. 15 (in English; summaries in English, Russian and Lithuanian).

А. Кришчюкайтис, Д. Дж. Вебб, А. Григалюнас. Неинвазивная оценка качества ооцитов рыб медака (Oryzias latipes) и определение их эмбрионального развития с помощью методов математической морфологии // Электроника и электротехника. – Каунас: Технология, 2009. – № 5(93). – С. 107–112.

Модели животных на ранней стадии развития используются для оценки загрязнения окружающей среды промышленными отходами. Эти модели применяются и при исследовании токсичности материалов, а также химических препаратов, употребляемых в производстве одежды и пищевых продуктов. Исследования (на базе животных моделей) могут раскрыть возможности выживания отдельных видов животных в процессе мирового изменения климата. Данное исследование показывает как современные методы цифровой обработки изображений (математическая морфология) могут быть использованы при оценке качества ооцитов (исследуя искусственно вызванную реакцию коры) пресноводных рыб медака (Oryzias latipes), а также развития седечно-сосудистой системы эмбриона. Оптоэлектрическая регистрация сердечной деятельности эмбриона, морфологический и временно-частотный анализ сигналов позволили выполнить детальный анализ сердечной деятельности. Совокупность этих методов может применяться для оценки нарушений развития эмбрионов рыб, как маркеров токсичности химических веществ, и для количественной оценки загрязнения окружающей среды. Ил. 5, библ. 15 (на английском языке; рефераты на английском, русском и литовском яз.).

A. Kriščiukaitis, D. J. Webb, A. Grigaliūnas. Neinvazinis medakų (Oryzias latipes) oocitų kokybės bei embriono vystymosi vertinimas matematinės morfologijos metodais // Elektronika ir elektrotechnika. – Kaunas: Technologija, 2009. – No. 5(93). – P. 107–112.

Ankstyvųjų vystymosi stadijų gyvūnų modeliai naudojami aplinkos taršai pramonės teršalais įvertinti. Šie modeliai taikomi ir drabužių bei maisto gamyboje naudojamu medžiagų ir cheminių preparatų toksiškumo tyrimams. Tokie gyvūnų modeliais pagrįsti tyrimai gali atskleisti atskirų gyvūnų rūšių išlikimo galimybes keičiantis pasauliniam klimatui. Šis tyrimas rodo, kaip šiuolaikiniai skaitmeninio vaizdų apdorojimo metodai (matematinė morfologija) gali būti panaudoti vertinant gėlavandenių žuvų medakų (Oryzias latipes) oocitų kokybę (tiriant dirbtinai sukeltą žievės reakciją) bei embriono širdies ir kraujagyslių sistemos vystymąsi. Optoelektrinė embriono širdies veiklos registracija ir signalo morfologinė bei laiko ir dažnio analizė leido atlikti detalią širdies veiklos analizę. Šių metodų visuma taikytina žuvų embrionų vystymosi sutrikimų, kaip aplinkos taršos bei cheminių medžiagų toksiškumo žymenų, detekcijai ir kiekybiniam vertinimui. Il. 5, bibl. 15 (anglų kalba; santraukos anglų, rusų ir lietuvių k.).