



FACULTY OF BIOLOGICAL SCIENCES

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Review of the doctoral dissertation of MTech. Manohara Mahadeva entitled “Membrane potential sets the tempo: Bioelectricity controls the rate of somitogenesis in chick embryos through mechanics”

A doctoral thesis of MTech. Manohara Mahadeva was undertaken in the Team of Reproductive Pathology and Translational Medicine at the Institute of Animal Reproduction and Food Research Polish Academy of Sciences under the supervision of dr hab. Magdalena Kowacz. The research presented in this dissertation was carried out thanks to funding obtained from the National Science Centre of Poland (grant number 2020/38/E/NZ3/00039).

The PhD thesis contents two already published original articles published in reputable scientific journals:

1. Mahadeva M, Niestępski S, Kowacz M. *Dependence of cell's membrane potential on extracellular voltage observed in Chara globularis*. *Biophys Chem*. 2024 Apr;307:107199. doi: 10.1016/j.bpc.2024.107199. Epub 2024 Feb 5.
2. Mahadeva M, Niestępski S, Kowacz M. *Modifying membrane potential synchronously controls the somite's formation periodicity and growth*. *Dev Biol*. 2025 Jan;517:317-326. doi: 10.1016/j.ydbio.2024.11.002. Epub 2024 Nov 8.

And one original article submitted to the journal *Developmental Biology*:

1. Mahadeva M, Niestępski S, Kowacz M. *Bioelectric control of tissue mechanics: Bioelectric control of tissue mechanics: Effect of membrane potential on somite deformability in chick embryos*.

MTech. Manohara Mahadeva is the first author of all three articles. He has a significant contribution to the articles, as declared in Annex No. 6. *Statement of the candidate on his substantive contribution to the creation of a work*. In all three cases, his contribution consisted of Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - Original draft, Writing - Review and Editing, and Visualization.





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The publications which constitute the main part of the dissertation are preceded by a “Vote of thanks”, “List of abbreviations”, “Abstracts” in English and Polish, “List of publications included in the doctoral dissertation” and Chapter 1. “Introduction”, containing subsections “General introduction and aims” and “Research hypothesis and objectives”, which outline the background of the research and its main assumptions.

The three articles that form the core of the dissertation are presented in three consecutive chapters numbered from 2 to 4. Their titles correspond to the titles of the manuscripts. The next two chapters of the dissertation, 5 and 6, are entitled “General discussion” and “Conclusions”. The dissertation also includes chapter 7, entitled “Implications and future perspective”, which presents preliminary research results that continue the topics raised in earlier chapters and directions for further research on the subject. The dissertation concludes with “References”, the required “Author statements” and “Published articles”.

The general aim of the PhD thesis was to understand the biological importance of extracellular and intracellular components of membrane potential, the influence of various stimuli on the formation of membrane potential and its significance for the course of early developmental stages, as well as to investigate how modifications in membrane potential affect the rate and mechanics of processes related to somitogenesis in chick embryos.

The first article (Chapter 2) presents the results of research concerning correlation between cell function, its membrane potential, resulting from uneven ion distribution, and microenvironment. This study asked the question whether the membrane potential of cells can be modulated by external stimuli such as CO₂. Research using *Chara globularis* as an experimental model and direct microelectrode technique has shown that elevated CO₂ level near the cell membrane alters its extracellular microenvironment, affecting its membrane potential (V_m). The study provided evidence for the existence of a negative extracellular voltage gradient (V_z) near the cell, as well as a coupling effect between V_z and V_m.

The study was expanded to include interesting analyses concerning the impact of elevated intracellular CO₂ on the potential of the external microenvironment. This approach has demonstrated that elevated intracellular CO₂ levels, resulting from cellular respiration processes, has no effect on the extracellular voltage suggesting that the metabolic activity of a cell can proceed without inducing changes in V_z.

The study also provides data on the biological significance and mechanism of formation of the zone of extracellular voltage (ZEV) associated with increased ion exchange in the spine and naturally damaged cells of using





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Chara globularis. The results confirm ZEV expansion near surfaces with enhanced ion exchange, which in the case of damaged cells may constitute, among the other things, a form of protection against pathogens.

The strengths of the research published in the first article include the methodological approach, such as the visualization of the extracellular voltage zone using microspheres of various diameters. Also noteworthy is the consideration of appropriate controls, such as voltage measurements in an environment with reduced pH due to HCl, which enabled the finding that the increase in negative extracellular voltage is the result of charge separation at the zone, not a change in the pH of the external solution.

In the second article (Chapter 3), the PhD candidate explores the correlation between the periodicity of somite formation and growth and the dependence of these processes on membrane potential. Chick embryos were used as a model in this study. PhD candidate analysed the effect of elevated CO₂ and KCl levels in the external environment of somite-forming cells on their membrane potential. Research has shown that both factors contribute to the depolarisation of these cells and may contribute to defining their V_m.

In the next step, it was examined how changes in V_m, achieved by modifying the microenvironment of somites, in terms of CO₂ concentration, affect the pace of somitogenesis. Data obtained by the PhD candidate shows that depolarization, induced by the presence of elevated CO₂ concentrations near the somite, accelerates somitogenesis, while hyperpolarisation reduces its pace. Studies have also shown that somite maturation is accompanied by their hyperpolarisation, and the observed changes in membrane potential are step-like between specific groups of somites. It has been demonstrated that the somite formation periodicity (related to cell migration and self-assembly) depends linearly on membrane potential, while the somite growth rate (related to cell proliferation) depends exponentially on membrane potential.

As in the first article (Chapter 2), an adequate research methodology was used and appropriate controls were ensured to exclude changes in pH of the extracellular environment as the trigger of the observed V_m changes.

Despite careful description and presentation of the results in the publications, there were minor flaws that do not affect their overall value. For example, in the first article (Chapter 2), there is an incorrect labeling/description of the panels in Figure 5 (i.e., the microscopic image is labeled as B, but should be A). In the second article (Chapter 3), in the materials and methods section, in the description of the potential-sensitive probe, DiBAC4(3), it is stated that “as the intensity of fluorescence increases, the cell becomes more hyperpolarized”, although an increase in the fluorescence intensity of cells indicates that these cells are more depolarised. Moreover, in the second article (Chapter 3) no information was provided on statistical tests used to evaluate the effect of stimuli on somite segmentation time and somite growth rate (Figure. 3.S2.).





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After reading the second article (Chapter 3), certain questions arise. Here are some of them: in order to compare the effect of different stimuli on a given group of somites, the “Vm of selected representative somites from each group was measured and averaged for any experimental conditions”. What exactly does this mean? Were the measurements taken for one (if so, which one) somite from each group and the results of these measurements averaged? Or was the Vm of each somite in a given group measured and averaged to obtain the average result for the entire group?

Can the presence of particles (microspheres) in the experimental setup interfere with the membrane potential of somites in any way? The non-charged microspheres used in the experiment should theoretically not affect the cell membrane potential. Has their lack of impact been confirmed? Were measurements of membrane potential taken in the presence of particles? Were they used exclusively to study the exclusion phenomenon resulting from the presence of an electric field (V_z) near the surface of the somites?

Were microscopic observations of small diameter microspheres (0.2 μm) performed under controlled conditions (i.e. at 5% CO_2)?

Were observations made of the exclusion of microspheres in the presence of a depolarising stimuli along the anterior-posterior axis of the embryo? The description of the experiment, “the exclusion of microspheres”, indicates that the embryos were stained with a potential-sensitive dye to mark the surface of the somites. Could this have affected the determination of the width of the particle exclusion zone?

The third article (Chapter 4), as yet unpublished manuscript, was devoted to the topic of changes in the mechanical properties of tissue accompanying its maturation. The study examined the influence of membrane potential on deformability of somite-forming cells during early embryonic development. The assessment of deformability concerned somite cells exposed to external stimuli (CO_2 and KCl) modulating their membrane potential. These analyses have established that depolarisation results in somite-forming cells becoming softer, while hyperpolarisation increases their stiffness.

Chick embryos were used in the experiments, and the micropipette aspiration technique was applied to assess somite deformability. Measurements of the deformability of somite-forming cells (expressed as aspiration length) were performed along the caudal-to-rostral axis, which showed that their deformability decreased exponentially with somite stage along the body axis of the embryo. Through measurements in the presence of cell depolarising stimuli, elevated concentrations of CO_2 and KCl with respect to control conditions, it was also established that the deformability of all somites increased. Furthermore, it has been demonstrated that under conditions that promote





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hyperpolarization, induced by reducing the concentration of CO₂ or KCl in the vicinity of the somite surface, the deformability of the somites decreases.

The research led to the conclusion that the increase in the stiffness of somites, i.e. their increasing resistance to deformation, is linked to their progressive hyperpolarisation, which in turn accompanies the maturation of somites as they reach more developmentally advanced stages, while induced depolarisation softens the somites. An increasing intracellular biomass concentration was identified as one of the causes of this phenomenon, which is related to the increase in the number of cells in the somite.

The results presented in the study are supplemented by the hypothesis of a possible mechanism through which V_m alters the stiffness of somites in chick embryos, suggesting that cell stiffness may be affected by changes in V_m through alterations in cytoskeletal structure. This involves changes in intracellular pH affecting actin polymerisation, whereby hyperpolarisation of the cell leads to a reduction in pH, which in turn facilitates actin polymerisation, thereby increasing cell stiffness. Furthermore, the PhD candidate suggests that cytoskeletal polymerization state may influence signalling pathways regulating somitogenesis, such as Wnt and Notch.

The strengths of this part of the dissertation include careful planning of experiments, which allowed to draw interesting conclusions. Also noteworthy is the mature manner of discussing the results obtained and a very good knowledge of the field, allowing for the formulation of reliable hypotheses concerning the implications of the findings.

After reading the third article (Chapter 4), certain questions arise. In this part of dissertation, the PhD candidate claims that “no noticeable alterations in overall somite volume were observed in response to induced V_m modifications, but clear changes in somite stiffness were observed”. How were the observations/assessments of somite volume made?

An interesting issue addressed by the PhD candidate in Chapter 7, “Implications and future perspective” is the correlation between knowledge about the influence of membrane potential and the expression of genes involved in the control of the segmentation clock and somitogenesis. This raises the question of whether in any further research plans related to the topic of the dissertation, would it be possible to compare the transcriptomic profiles of individual somites/groups of somites that would be exposed to various factors affecting membrane potential, as examined by the PhD candidate?





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The results obtained by the PhD candidate are characterised by their originality and provide a wealth of interesting and valuable scientific data. The candidate was the first to describe the pattern of membrane potential changes along the axis of a vertebrate embryo. Among other things, the influence of membrane potential on the pace of somitogenesis and its significance as a factor modulating the mechanical properties of somite-forming cells in the chick embryo has been demonstrated.

The high quality of the results obtained in all three manuscripts presented in the dissertation is ensured by well-chosen and diverse research methods, careful design of experiments with appropriate controls, which also demonstrate that the PhD candidate has acquired the skills of a mature scientist.

All three articles are closely related, and the results presented in each of them are logically connected and reflect a well-thought-out and excellently implemented research plan. The choice of research methodology, the manner of data presentation, their interpretation and discussion demonstrate not only the scientific maturity of the PhD candidate, but also his readiness to take on further scientific challenges.

As stated above, the results presented in the dissertation are extremely interesting and contribute to expanding knowledge about the role of membrane potential in the development and functioning of organisms. Reading the dissertation was not only a pleasure, but also raised several questions. Below are some of them for the purpose of scientific discussion during the dissertation defence:

1. Regarding the first and second article (Chapters 2 and 3), could the PhD candidate comment on why was no statistical test used to compare means of groups with regard to the data obtained from experiments concerning the effect of external stimuli on the extracellular membrane potential zone (article 1, Chapter 2) or the effect of CO₂ and KCl on the membrane potential (V_m) of somite-forming cells in chicken embryos (article 2, Chapter 3)?
2. Regarding the second article (Chapter 3), is it possible to predict whether and what developmental changes (apart from changes in the pace of somitogenesis) will be caused by external factors influencing the membrane potential of somites?
3. Regarding the second and third article (Chapters 3 and 4), the PhD candidate suggests a close relationship between changes in V_m of somites and structural changes in cellular components, including the actin cytoskeleton. Would it be possible to confirm this assumption and, at the same time, examine the dynamics of the actin cytoskeleton in somite-forming cells under experimental conditions presented in the dissertation, using, for example, SIR-actin fluorescent probe for live-cell microscopy? Could this approach help to





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- determine the role of the actin cytoskeleton in the dynamics of somite cell responses to induced depolarisation and hyperpolarisation?
- Regarding third article (Chapter 4), what is the PhD candidate's opinion on whether it would be worthwhile to test the hypothesis concerning the Vm-dependent state of polymerisation of the actin cytoskeleton on the functioning of signalling pathways navigating somitogenesis, for example by performing transcriptomic studies at the level of somite-forming cells exposed to various stimuli promoting membrane depolarisation and hyperpolarization?
 - The results obtained by MTech. Manohara Mahadeva are not only of great cognitive value, but also have interesting practical implications related to medical aspects. Depolarised membrane potential, as the PhD candidate himself points out, is a common feature of processes such as embryogenesis, tissue regeneration and tumorigenesis. Therefore, in light of the results obtained, the question arises as to whether and how the PhD candidate perceives the possibility of manipulating the membrane potential of cells as a potential therapeutic approach in the treatment of cancer.

I rate the doctoral thesis by MTech. Manohara Mahadeva very positively. I hereby declare that the doctoral thesis meets the requirements specified in Polish law, i.e.:

- demonstrates general theoretical knowledge in the disciplines of animal science and fisheries, particularly in the field of biophysics of biological membranes and the role of membrane potential in biological processes related to embryonic development;
- confirms the PhD candidate's ability to conduct independent research;
- provides an original solution to the scientific problem of the influence of various stimuli on the formation of membrane potential as well as its significance for the course of somitogenesis.

According to above statements, I conclude that the doctoral dissertation of MTech. Manohara Mahadeva meets the conditions specified in Article 187 of the Act of July 20, 2018 Law and Higher Education and Science (i.e. Journal of Laws of 2024, item 1571, as amended), and I request the Scientific Council of the Institute of Animal Reproduction and Food Research Polish Academy of Sciences in Olsztyn to admit MTech. Manohara Mahadeva to further stages of the proceedings for the conferment of the doctoral degree in the field of Agricultural sciences disciplines of animal science and fisheries.

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