University of Hildesheim – Department of Biology
Marienburger Platz 22 – D-31141 Hildesheim – Germany

Prof. Dr. Bohuslav Gaš
Dean
Faculty of Science
Charles University in Prague
Albertov 6
128 43 Prague 2
Czech Republic

Prof. Dr. Uwe Kierdorf

Department of Biology

Phone: +49 5121-883-917
Fax: +49 5121-883-911
e-mail: uwe.kierdorf@uni-hildesheim.de

Secretary:
Susanne Ohlendorf
Mo 07.30 – 16.00 h
Tu, Th 12.30 – 18.00 h
We, Fr 07.30 – 12.30 h
Phone: +49 5121-883-910
e-mail: ohlendor@rz.uni-hildesheim.de

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Review of the Ph.D. thesis by Mgr. Erika Kužmová "Hormonal Aspects of Antler Growth Regulation"

The antlers of deer are unique biological structures since they are the fastest growing bones in the animal kingdom and the only mammalian bony appendages capable of complete regeneration. Recently accumulated evidence indicates that antler regeneration is a stem-cell based process that involves the periodic activation of mesenchymal stem cells located in the pedicle periosteum. The finding that deer are able to regrow a huge bony structure via a stem cell-based process makes the antlers a highly interesting subject for study that may provide insights valuable for reaching the ultimate goal of regenerative medicine, namely to stimulate the regrowth of amputated limbs in humans.

The thesis submitted by Erika Kužmová addresses two central issues of current antler research, namely, the characterization of the stem cells involved in antler regeneration and the hormonal factors stimulating antler growth *in vivo* and the proliferation of "antler stem cells" *in vitro*.

The thesis consists of an introductory chapter and four papers, two of which have already been published by international, peer-reviewed journals, one is in press, and one has been submitted to an international journal. Erika Kužmová is the first author of three of the four papers.

The brief introductory chapter gives necessary background information on the function of antlers, the annual antler cycle, the ontogenetic development of pedicles and antlers and the basic hormonal factors involved in the control of antler growth and mineralization. The author subsequently summarizes the different aspects that make antlers an interesting subject for study, highlighting in particular the biomedical applications that may be derived from a better understanding of the factors and processes involved in the control of antler growth. Consideration is then given to potential limitations of antler studies, related not the least to the large size of deer, the lack of specific antibodies and the insufficient characterization of the "antler cells" used in many studies. The need to use defined cell populations for *in vitro* studies is rightly highlighted. The introductory chapter provides a concise, well-balanced overview about some basic facts of antler development and the hormonal control of antler growth, thereby setting the scene for the author's own contributions. The pertinent literature in the respective fields is covered and appropriately discussed.

The first paper, published in the peer-reviewed online journal *PLoS ONE*, analyzes the effects of antler growth stage, male individuality, and culture conditions (primary vs passaged cultures, concentration of fetal calf serum), on the proliferation of mixed red deer antler cells *in vitro*. The results indicate a significant effect on cell proliferation of all factors except antler growth stage. The observation of a significant effect of the factor "individuality" seems especially important, since it has not been considered in previous studies. Future studies will have to show which developmental and/or physiological factors are connected to "individuality".

The second paper was presented at the 7th International Deer Biology Conference in Chile and published after peer review in a supplemental issue of the journal *Animal Production Science.* Analyzed were the effects of different culture conditions on the percentage of cells positive for the cell surface antigen STRO-1, a marker of mesenchymal stem/progenitor cells, that could be obtained from antler and pedicle tissue cultures. The mixed cell cultures were obtained from the antler growth region, antler periosteum and pedicle periosteum of male red and fallow deer. The percentage of STRO-1^+ cells in the cultures varied widely (between 0.4 and 39% for fallow deer and between 1.8 1nd 16.5% in red deer). While no significant influence of the sampling site was found (an unexpected result), the influence of passage was significant, with the highest percentage of STRO-1^+ cells obtained from the second passage cultures. For reasons yet to be explained, opposite effects of the type of culture (mixed *vs* STRO-1^-) were obtained for fallow deer and red deer. The results show a strong effect of the culture conditions on the potential yield of STRO-1^+ cells that needs to be considered in future studies. The fact that the authors were able to cultivate STRO-1^+ cells from repeatedly sorted STRO-1^- cultures raises the important question of the origin of the positive cells. Different possibilities for this are discussed, thereby identifying this issue as a field requiring further study.

The third paper is a manuscript that has not yet been published, but is currently under review. The study analyses the effects of different concentrations of IGF-1 and sex steroids, given either alone or in combination, on the proliferation rate in mixed cell cultures obtained from the growth region of red deer antlers at different times after casting of the previous set of antlers. The results were quite heterogeneous and sometimes, as e.g. regarding the effects of estradiol or the antiandrogen cyproterone acetate, did not show a general trend. A potentially important finding is that, contrary to previously reported results of other groups, IGF-1 did not stimulate cell proliferation while testosterone at least in some of the treatments did. From their findings the authors conclude that testosterone probably stimulates antler cell proliferation *in vitro*, but that the effects may be antler stage-dependent. Interestingly, in the study of *in vitro* antler cell proliferation in cultures not treated with sex steroids (paper 1) antler growth stage was not a significant factor. Clearly further studies are needed to clarify the role of sex steroids, testosterone in particular, on the different types of antler cells. Based on the results of this paper, it will certainly be possible to perform future experiments in a more focused form.

The fourth paper, which will be published in a forthcoming issue of *Frontiers in Bioscience* is a comprehensive, in-depth review of the role of sex steroids, testosterone in particular, and IGF-1 on the behavior and the antler cycle of deer, using data from *in vivo* and *in vitro* experiments. The paper gives a balanced overview about the views of previous workers and undertakes an interesting meta-analysis of studies analyzing the effects of testosterone and IGF-1 on antler cell proliferation *in vitro*. In discussing the outcome of this meta-analysis, which shows a significant trend for a stimulatory effect of IGF-1 but not of testosterone, but high measures of heterogeneity for both factors, the authors rightly highlight some problems of the studies conducted so far. A major problem is that the "antler cell cultures" used in these studies are ill-defined, probably constituting a mixture of mesenchymal cells, chondroprogenitors, chondrocytes and cells of the osteogenic lineage. Already slight variation of the biopsied regions

will probably results in marked changes of the percentages of these cell types in the cell culture. There is reason to assume that the proliferative behavior and their reaction to testosterone and IGF-1 vary widely among these different cell types. Therefore the authors rightly state that the methodology of future studies needs to be standardized to achieve meaningful results. The authors then discuss a number of *in vivo* studies addressing the same question. Especially the outcome of a "double-castration" experiment (castrated fallow bucks additionally treated with the antiandrogen cyproterone acetate) suggests that a minimum level of testosterone action is required for antler growth. This thorough review will without doubt be a highly useful starting point for those endeavoring to study the hormonal regulation of antler growth in the future. The paper clearly identifies the weak points in previous studies and the fields needing special consideration in future work.

The thesis submitted by Erika Kužmová is a valuable contribution to antler research that both critically evaluates previous work by other authors and contributes very interesting new findings from her own experiments. Regarding the *in vitro* experiments, the results clearly demonstrate the necessity to take into consideration a number of factors that tended to be neglected in previous studies. On the one hand this of course complicates the overall picture, but on the other hand it shows up ways of how to increase our understanding of the control of antler growth. Future *in vitro* studies will need to be more specific especially with regard to the selection and the characterization of the cultured cell population and the (differential) survival and proliferation of the different cell types under varying culture conditions, and this insight we owe not the least to the work presented by Erika Kužmová.

In conclusion, I consider the thesis submitted by Erika Kužmová suitable for defence and to be of a quality that fulfils the criteria for obtaining the Ph.D. degree.

Prof. Dr. rer. nat. habil. Uwe Kierdorf