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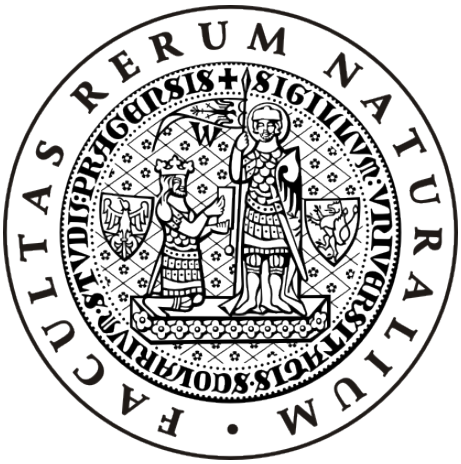

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Hormonal Aspects of Antler Growth Regulation

Doctoral thesis

Supervisor: Prof. Ing. Luděk Bartoš, DrSc.

Prague, 2011

Declaration:

Hereby I declare that I compiled this thesis independently, using only the listed literature and resources. I also declare that the presented work was not used to acquire any other academic degree.

Prague, 30.5.2011 Erika Kužmová

Antlers, such a beautiful and fascinating burden ...

Acknowledgements

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Contents

Abstract

Deer antlers are the only mammalian organ that completely regenerates and therefore they became an object of rising interest as a potential model for bone growth and development. In recent years, it has been confirmed that annual regeneration of the antler is initiated from the stem cell niche localised in the pedicle periosteum. Antlers grow to the length at the tip. Only a little is known about endocrine stimulation of antler growth and some discrepancy has arisen between *in vivo* and *in vitro* studies over the decades. As the secondary sexual character, the antler cycle timing and growth are linked to seasonal levels of testosterone. Since the levels are at their minimum during the antler growth phase, according to many mainly *in vitro* studies, insulin-like growth factor-1 (IGF-1) tends to be accepted as the "antler stimulating hormone".

Since the conclusion about the role of IGF-1 was contradictory to previous opinions and also in contrast with our own experience, we aimed to verify the role of IGF-1 *in vitro*. Our experiments were based on existing *in vivo* studies demonstrating the importance of testosterone, even in its low levels, and on the hypothesis that testosterone should be the "antler stimulating hormone". We performed *in vitro* experiments on cells derived from the growing antler tips of the red deer (*Cervus elaphus*) at various antler growth stages. Within *in vitro* cultivations we studied the effects of different factors such as antler sampling day, male individuality, passaging, concentration of foetal calf serum (FCS) and length of the experiment on the intensity of the antler cell proliferation. We found that all these factors not only significantly influenced the cell proliferation, but depending on these factors the intensity of proliferative response of cells from different individuals or under hormonal treatments was significantly changed. Next we studied the effects of various hormonal treatments as testosterone, IGF-1 and estradiol, as well as effect of antisteroids Cyproterone acetate, Flutamide and ICI 182,780, on antler cell proliferation. None of the treatments caused consistent proliferative response. However, testosterone and, partially, estradiol stimulated the proliferation in several cases. On the other hand, the stimulating effect of IGF-1 was not confirmed in our experiments, as IGF-1 either did not affect the antler cell proliferation or even inhibited it in some cases. We isolated STRO-1 positive mesenchymal stem cells from the mixed antler cell cultures but we could not perform hormonal experiments with the cells, as we were unable to obtain sufficient amounts of the positive cells for our experiments. Despite this, our results suggest that the sex steroids are mitogenic for antler cells *in vitro* and might play an important role in the stimulation of antler growth.

Our results are in accordance with many physiological and behavioural studies. They support the inevitable role of testosterone in the antler re-growth phase and suggest that the primary cultures may better represent the *in vivo* conditions and processes that occur in regenerating antlers.

Abstrakt

Parohy jeleňov sú jediným kompletne sa regenerujúcim orgánom u cicavcov a záujem vedcov o ich využitie ako modelu rastu a vývoja kostí stúpa. V posledných rokoch sa ukázalo, že regenerácia parohov je iniciovaná z kmeňových buniek lokalizovaných v okostici pučnice. Následný rast parohu do dĺžky však prebieha v rastovom vrcholčeku. Len málo sa vie o endokrinnej stimulácii rastu parohu a už dlhé roky existuje nesúlad medzi *in vivo* a *in vitro* štúdiami. Ako druhotný sexuálny znak sú parohy úzko späté so sezónnymi hladinami cirkulujúceho testosterónu. Keďže sú jeho hladiny najnižšie práve v čase rastu parohov a mnohé *in vitro* štúdie poukazujú na stimulačný efekt inzulínu podobného rastového faktoru (IGF-1), viacerí odborníci sa prikláňajú k názoru že IGF-1 je "hormón stimulujúci rast parohov".

Tento záver je ale v rozpore s výsledkami *in vivo* štúdií, ktoré ukazujú nevyhnutnosť testosterónu pre rast parohov aj v jeho nízkych koncentráciách, a taktiež s predchádzajúcim názorom, že testosterón by mal byť "hormón stimulujúci rast parohov". Zamerali sme sa teda na overenie účinkov IGF-1 na parožné bunky. Uskutočnili sme sériu *in vitro* experimentov na parožných bunkách izolovaných z viacerých štádií rastových vrcholčekov parohov jeleňa európskeho (*Cervus elaphus*). Počas *in vitro* kultivácií sme sledovali vplyv rôznych faktorov ako sú deň odberu tkaniva, individualita jedincov, pasážovanie, koncentrácia bovinného séra a dĺžka experimentu na intenzitu proliferácie parožných buniek. Zistili sme, že všetky tieto faktory signifikantne ovplyvnili proliferáciu buniek a dokonca sa vplyvom týchto faktorov menila intenzita proliferačnej odpovede buniek z jednotlivých jedincov, alebo na sledované hormóny. Bunky primárnych kultúr, kultivované v 10% bovinnom sére odobraté na 15. deň od zhodenia parožia proliferovali najintenzívnejšie. Ďalej sme sledovali účinky rôznych hormónov ako testosterónu, IGF-1 a estradiolu, ako aj účinok antisteroidov Cyproterón acetátu, Flutamidu a ICI 182,780 na proliferáciu parožných buniek. Žiadny z hormónov nevyvolával u buniek jednotnú proliferačnú odpoveď, hoci testosterón a čiastočne aj estradiol v niekoľkých prípadoch proliferáciu stimulovali. Naše experimenty však nepotvrdili stimulujúci účinok IGF-1. IGF-1 buď nemalo žiadny účinok, alebo proliferáciu vo viacerých prípadoch inhibovalo. Zo zmiešaných parožných bunkových kultúr sa nám podarilo izolovať STRO-1 pozitívne mezenchymálne kmeňové bunky. Žiaľ, pre hormonálne experimenty sa nám nepodarilo izolovať dostatočné množstvo týchto buniek. Napriek tejto skutočnosti, naše experimenty ukazujú, že pohlavné steroidy majú mitogénny vplyv na parožné bunky *in vitro*, a teda by mohli hrať dôležitú úlohu v stimulácii rastu parožia.

Výsledky, ktoré sme získali, sú v zhode s výsledkami mnohých iných fyziologických a behaviorálnych štúdií. Podporujú úlohu testosterónu vo fáze rastu parožia a ukazujú, že primárne kultúry pravdepodobne lepšie reprezentujú *in vivo* podmienky a procesy prebiehajúce v regenerujúcich sa parohoch.

Introduction to the Thesis

1 Introduction

Antlers have fascinated people since ancient times and prehistoric antlered deer paintings can be found in many European caves (Fig. 1). This is no wonder as antlers are an extravagance of nature, rivalled by few other biological luxuries as flowers, butterfly wings or peacock tail [1]. However, despite their exceptional growth and regeneration capabilities, little scientific attention has been paid to them. In the second half of the 20th century, highly regarded researchers such as Richard Goss, Zbignew Jaczewski, Anthony Bubenik, George Bubenik, Gerald Lincoln, Robert Brown and many others contributed to the field or even dedicated their lives to antler study. As Richard Goss stated in his outstanding monograph "Deer Antlers: Regeneration, Function, and Evolution", the study of antlers is a rewarding challenge, because in such an unexplored field as this, almost anything one learns is new discovery. Indeed, in the last decades interest in antlers as the only mammalian appendages capable of complete regeneration raised markedly and antlers attract not only zoologists and evolutionary ecologists but also researchers from various biomedical and pharmacological fields. The number of published scientific papers about antlers increases continually and this autumn "The 3rd International Symposium on Antler Science and Product Technology" will be held in China.

Still, almost after 30 years, Goss' words are relevant: "The mechanism by which these "bones of contention" grow and differentiate into such magnificent morphologies is a source of wonder and curiosity."

Figure 1: Sketch of a deer in The Cave of La Pasiega in Spain. By José-Manuel Benito Álvarez.

1.1 Antlers and Their Function

Antlers are a luxurious example of the secondary sexual characters unique to cervids. These cranial bony appendages are typical for males, but can be found also in females of reindeer *Rangifer tarandus*, or initiated in females of other deer species when administrating testosterone [2].

The original function of antlers is not known, and there is some controversy in what function was the primary and which ones were the secondary [1, 3, 4]. While some believe that the antlers developed primarily as weapons [3], paleontological findings indicate that antlers developed first as soft non-mineralised persistent appendages serving more as display and probably scent-dispensing organs than weapons. Only after antlers become mineralised do they serve as weapons in intraspecific male competition [4]. The annual renewal of antlers appears as the compensation of frequent breakages after aggressive encounters or as the adaptation to temperate zones preventing necrosis of frozen ends [5]. The antlers gain also other secondary functions and may be used for many purposes. They enable reindeer to find vegetation underneath the snow [6] or other deer species from the trees [7]. The elaborate palm structure of moose antlers may act as a parabolic reflector and enable moose males to better locate calling females [8]. The abundance of sebaceous glands in the velvet (specialized antler skin) of antlers supports their function as olfactory projectors. As antlers are richly vascularised and almost hot to the touch during the growing velvet period and the branched configuration increases the surface area, they might serve as thermal radiators during summer when males increase their metabolism to fortify themselves for the upcoming rutting period [9].

Regardless of their function, annual antler re-growth represents an incredible nutritional demand for deer and their development is associated with pathogen resistance, thus representing an honest signal of genetic quality [10]. Not surprisingly antlers play a major role in the social life of deer and serve as "social semaphores". They help to establish the rank order, obviate intraspecific conflicts since male combat may cause serious wounds, and not least they serve as intersexual display. Moreover antler size plays a significant role in sexual selection as an indicator of individual quality [11, 12]. As secondary sexual characters, antler growth is closely related to circulating levels of testosterone and both are modulated by other hormones, social position and agonistic behaviour [13, 14].

1.2 Antler Development and Annual Cycle

Antlers grow out from permanent extensions of the frontal bone called pedicles. Pedicles start to evolve during early prenatal development in males but disappear in the later prenatal stages [15]. Later at the time of puberty the males under the influence of testosterone, develop pedicles and start growing primary antlers. In red deer (*Cervus elaphus*), the primary and the later regenerated antlers are cast in spring and antler re-growth starts immediately (Fig. 2). In the next three months the antlers grow and elongate at the most spectacular rate in animal kingdom - up to 1cm per day on average. Moreover if one combines the rates of elongation of the

several tines growing simultaneously on both antlers, the production is as much as 10 cm of new antler material every day in the midseason. Such astonishing growth requires similar growth velocity of nerves and blood vessels and exaggeration of the normal mineral metabolism in the body to mobilise the vast quantities of calcium and phosphorus deposits each year into the regenerating antler [1]. For example, antlers of a 200 kg adult red deer may weight 30 kg [16]. After the rapid growth phase lasting approximately 100 days, antlers remain in velvet until they fully mineralise. In the late summer the velvet shedding begins and antlers are "ready" for the rutting period. Hard bony antlers are cast again in the spring and new antler re-growth follows.

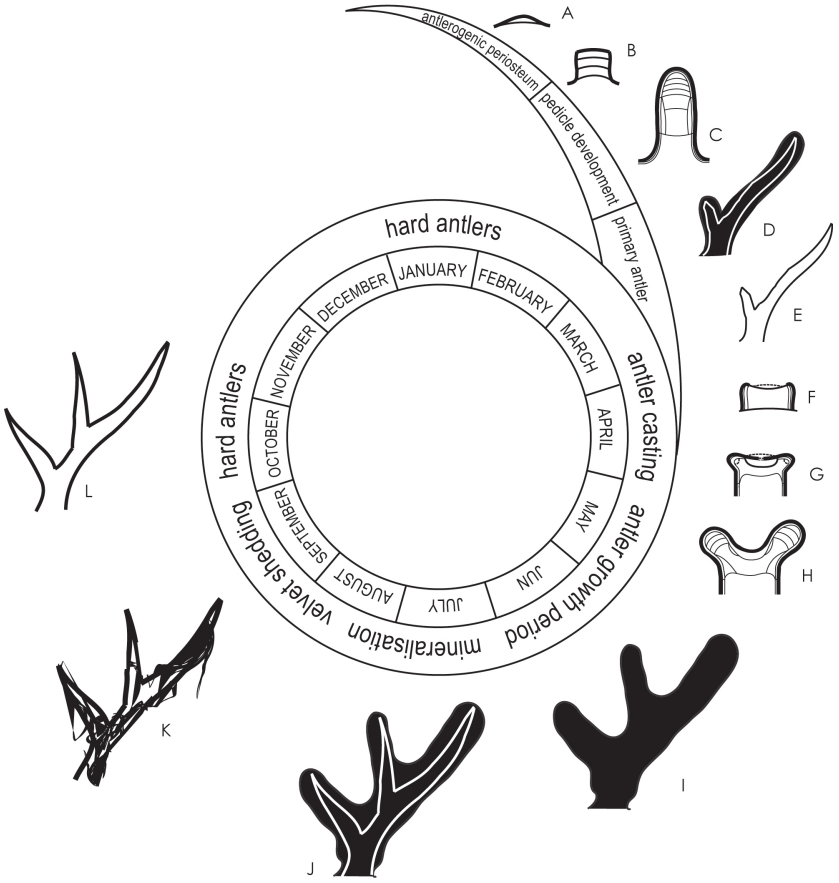

Figure 2: Development of the pedicle from the antlerogenic periosteum, the primary antler and the annual antler cycle. (A) antlerogenic periosteum as a thickening of the periosteum of the frontal bone, (B) development of the pedicle, (C) development of the primary antler, (D) primary antler in velvet, usually unbranched, (E) mineralised primary antler, (F) pedicle on the antler casting day "the stage of oil lamp bowl", (G) formation of the growth centres "the millstone-like structure", (H) formation of the main beam and the brow tine "small saddle stage", (I) branched velvet antler, (J) fully mineralised velvet antler, (K) velvet shedding, (L) hard antler. A, B, C, F, G, H adapted from Price et al. [16]

1.3 Pedicle and Antler Origin

Both pedicles and antlers are derived from so called "antlerogenic periosteum" overlying the frontal bone. The origin of antlerogenic periosteum has not been experimentally shown yet, but is likely to be neural-crest-derived as are other skull bones [17]. Even more, due to its remarkable capacity for self-differentiation and the fact that the cells contain abundant glycogen, the antlerogenic periosteum resembles a piece of post-natally retained embryonic tissue as noticed by Li and Suttie [15]. However, the expression of embryonic stem cell markers in antlerogenic periosteum has not been studied so far [18]. A transplantation of the antlerogenic periosteum onto the foreleg or forehead causes a pedicle and antler development at these sides [15]. On the other hand, recently it has been demonstrated that transplantation of pedicle periosteum cannot initiate antler development and its function is restricted to antler regeneration [19].

Primary antler growth and annual antler re-growth are initiated from a stem cell niche localised in the pedicle periosteum [20–22]. Progenitor cells isolated from pedicle periosteum as well as from the growing antler tip express markers of undifferentiated cells and differentiate along osteogenic, chondrogenic and with antler tissue unrelated adipogenic lineages *in vitro* [21]. Developmental signalling pathways involved in the control of skeletal development and regeneration in other vertebrates were also shown to be involved in antler regeneration [17].

1.4 Process of Regeneration and Structure of the Growing Antler Tip

As mentioned above, antler re-growth starts by activation of the stem cell niche localized in the pedicle periosteum [18, 21]. Prior to antler casting, these cells form a swollen rim around the distal pedicle [23]. At this place the osteoclast activity is the most intense and antler casting is initiated. After antler casting the exposed casting surface of the pedicle is rapidly covered by a migrating epidermis. The wound healing and formation of the antler bud and future growth centres occur very rapidly [1]. The morphological stages of the initial antler regeneration are nicely described in Chinese [23]. Immediately after antler casting, blood is retained in the depressed central top of the casting surface resembling a bowl. This stage is called "the stage of oil lamp bowl". The early wound healing stage occurs one or two days after antler casting, when the blood dries and a scab is formed. This stage is called "tiger eye stage". Once the diameter of the scab becomes smaller a "millstone-like structure with an axle", the scab, located in the centre is created. Finally, formation of the main beam and the brow tine is called "small saddle stage" and after the bez tine is created, the structure is called "the stage of silver ingot" [23].

Antler growth occurs at the antler tip. The growing tip is divided into zones [16] (Fig. 3). Under the velvet, the fibrous perichondrium is localised. This is followed by an intensively proliferating progenitor cell layer of reserve mesenchyme responsible for growth in length. Cells isolated from the mesenchymal zone have extended life span *in vitro* since they can be grown for over 80 passages and for

up to 10 months in culture before they stop dividing [24]. Under the mesenchymal zone, the prechondroblastic zone is situated. Cells in these zones start to arrange into longitudinal columns and are richly vascularised. Further proximally, the chondrocytes undergo maturation and the cartilage matrix is mineralized. During the special form of endochondral ossification, the mineralized cartilage is resorbed and completely replaced by bone [18].

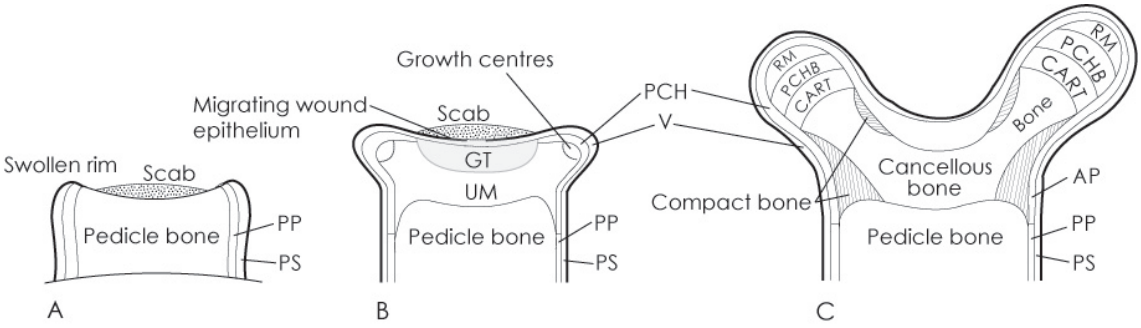

Figure 3: Antler growing tip development and zones. (A) pedicle on the antler casting day and swollen rim presence around the edge, (B) formation of the growth centres and of the scab, approximately 10 days after hard antler casting, (C) formation of the main beam and the brow tine, approximately 30 days after hard antler casting. PS-pedicle skin, PP-pedicle periosteum, PCH-perichondrium, V-velvet, GT-'granulation' tissue, UM- undifferentiated mesenchyme, AP-antlerogenic periosteum, RM-reserve mesenchyme, PCHB-prechondroblasts, CART-cartilage. Adapted from Price et al. [16].

1.5 Hormonal Regulation of the Antler Cycle

Since antlers play an important role in the social interactions of deer during the breeding time, their cycle is linked to the seasonal fluctuation of sex hormones which is regulated by changing day length [24]. Though regeneration of antlers is a complex process regulated by environmental and systemic factors, they show endogenous rhythms [1]. The function of other hormones as $1.25(OH)_2D_3$, thyroid hormones, cortisol and prolactin associated to the antler cycle are only poorly understood [16].

Generally, it is accepted that sex steroids, particularly testosterone, are required for pedicle and primary antler development and are the most important for the timing of the annual events in the antler cycle. While high levels of testosterone cause antler mineralization and velvet shedding in the late summer, their rapid decline below distinct threshold values during springtime cause antler casting [25, 26]. Castration of a calf prevents pedicle and primary antler development. Castration during the hard antler period causes a drop in testosterone levels and premature antler casting. Castration during the velvet period will delay velvet shedding and prevent full mineralisation of the antlers [16]. There is evidence that antlers of castrates are in fact benign tumours [18] and roe deer will react more massively than the other species by developing so called "peruke" [27].

During antler re-growth, systemic levels of testosterone are at their minimum and deer males are considered as almost "functional castrates" [24, 28]. Hence many authors have assigned only a minor role to testosterone in this phase, but this has been a matter of controversy over the decades [13, 29, 30]. Based on several *in vivo* and *in vitro* studies [28, 31–37] IGF-1 has become widely accepted as the "antler stimulating hormone" [24, 38]. However there exists sufficient evidence speaking for the need of low concentrations of testosterone for the stimulation of the antler regeneration [13]. Nonetheless still only a little is known about the endocrine stimulation of the early stages of antler re-growth, about the activation of the antler progenitor cells in the pedicle periosteum and their proliferation and differentiation into an antler bud [17].

1.6 Why to Study Antlers

Deer antlers are remarkable creations of nature offering an opportunity to study basal mechanisms of behavioural regulation of an honest secondary sexual character directly representing the hormonal background of the owner. Furthermore, they provide a unique model for studying developmental processes and complete regeneration of a complex bony organ in mammals [17]. As antler regeneration does not depend on innervation or direct contact between wound epithelium and mesenchymal tissue, they moreover demonstrate that the regeneration of a large bony appendage in mammals can be achieved by a different process as is the epimorphic regeneration in lower vertebrates [18]. Understanding of the underlying mechanisms may provide information to design therapeutic strategies for the diseased or damaged human tissues and help to elucidate why regeneration is limited in other mammals [16]. Recent findings of stem cell based origin of antler regeneration make antler regeneration even more relevant as a model for human bone, nerve and vascular regeneration [18, 21, 39–43]. It is worth mentioning that in addition to the rapid growth the antler innervating neurons show other remarkable characteristics as an amazing neuron survival after repeated axotomy and the ability to re-enter the growth/regeneration stage every year after more than 8 months of denervation [43].

The potential biomedical applications of antlers are far-reaching. Recently, establishment of a new stem cell line from antlerogenic cells and successful xenoimplantation of these cells has been reported [44, 45]. Antler bone has been used as a suitable scaffold material for bone tissue-engineering and bone reconstruction [46]. Last but not least, deer antler velvet is a promising farmacological product which has been used by Oriental cultures for thousands of years. Antler velvet has long been used as a traditional medicine for relieving pain, to combat aging, increase energy, stimulate muscle growth or enhance sexuality. It is believed to have anti-inflammatory, anti-cancer, immune stimulative and pro-growth effects, but not all of them have been experimentally proved yet [47, 48].

Another important fact to mention is that due to castration antlers develop tumour-like structures permanently covered in velvet. The answer to the question of why antlers develop benign tumours in the absence of sex steroids and appear

resistant to malignant cell transformation might have important implications for cancer biology [18].

1.7 Antler Study and Its Limitations

Study of antlers in general brings some difficulties along the way. The seasonal nature of antler growth limits the number of experiments that can be undertaken each year. The fact that deer is not a "mainstream" organism reduces the commercially available deer specific antibodies for immunohistochemical and immunocytochemical studies and limits the usage of molecular methods. However, the recent announcement of the sequencing of a substantial portion of red deer genome by researchers from New Zealand is a milestone for the deer industry and may bring benefit also to antler research. The utilization of deer as a model organism is however limited not only by difficulties of potential genetic manipulations but also by the demands of extensive *in vivo* experiments. To overcome these limitations, the xenograft approach of deer tissue transplantations into nude mice (Fig. 4) has the potential to become an appropriate tool to study the underlying mechanism of antlerogenesis and organogenesis/regeneration in general, although more research is required to further develope this model [49].

Figure 4: A pedicle-shaped protuberance (arrow) formed from the subcutaneously transplanted antlerogenic periosteum on a nude mouse head (from Li and Suttie [15], with permission of the author).

The more advanced knowledge and methods about bone development and regeneration processes in other model organisms together with our desire for deeper understanding of the underlying mechanisms of antler regeneration draw our attention to signalling pathways and local molecules involved in antler regeneration. Hence less attention is paid to the hormonal regulation and physiological mechanisms of antler re-growth although one of the most important questions yet to be satisfactorily examined is the identification of the "antler stimulating hormone" or "antler growth stimulus". Here there is no unified opinion whether testosterone or

IGF-1 is the main factor responsible for antler growth and the discrepancy is mainly between the *in vivo* and *in vitro* reports. While the majority of the *in vivo* studies support the role of testosterone [13, 29, 30], many of the *in vitro* studies show the mitogenic effect of IGF-1 on antler cells [32–36]. However, there are several issues related to the *in vitro* cultivation of antler cells. First, there are several factors that might modify the proliferative response of the antler cells *in vitro* and which have not been satisfactorily investigated in the existing literature. Second, all *in vitro* hormonal studies were performed on mixed antler cell populations containing different types of progenitor mesenchymal cells, chondro- and osteo-progenitors, chondroblasts, or even osteoblasts.

One way to overcome some of the above mentioned problems would be to use the defined cell populations instead of mixed antler cell populations. Such attempts were for the first time performed by Rolf et al. [21, 22] and resulted in isolation of "pure STRO-1+ mesenchymal stem cell cultures" derived from pedicle periosteum or regenerating antler tip. However, surprisingly high numbers of these cells could be isolated (up to 38% from fallow deer cultures and 16.5% from red deer cultures). One has to face problems connected to maintaining the undifferentiated state of the isolated cells as well as obtaining sufficient amounts for extensive hormonal experiments.

2 Aims of the Study

The presented work deals with the *in vitro* experiments on cells derived from the growing antler tips at various antler growth stages. The aims of this study were:

1. **To investigate factors influencing antler cell proliferation *in vitro*.** Among existing studies, we found out that some factors were not satisfactorily investigated, but might influence the proliferative response of antler cells. The important ones in our opinion were: tissue sampling date, deer individuality and factors of culture conditions such as effect of foetal calf serum concentration, passaging or length of the hormonal treatment.

2. **To examine the proliferative response of mixed antler cell populations to hormonal and growth factor treatments (particularly to testosterone, estradiol and IGF-1) alone or in the co-treatment with antiandrogens cyproterone acetate and flutamide and antiestrogen ICI 182,780 under varying experimental design.**
   If one of the treatments is the "antler stimulating hormone", its mitogenic effect should be present in all culture conditions and identically in cell cultures of all sampling days.

3. **To isolate mesenchymal stem cells out of mixed antler cell cultures and to perform hormonal experiments with "pure" antler cell populations.**
   If antler renewal is caused by re-activation of stem cells in the pedicle periosteum and the antler growth is localised to the growing antler tip, where the progenitors proliferate, then the hormonal experiments performed on such cells would be of high significance in answering the question of "antler stimulating hormone".

3 Conclusions

Our experiments confirmed the significant effect of factors antler sampling day, male individuality, passaging, foetal calf serum concentration and length of the experiment (hormonal treatment) on the antler cell proliferation *in vitro*. The cultivation factors, mainly passage, also significantly influenced the number of stem cells obtained from the mixed antler cell cultures.

The proliferative response of antler cells to hormonal treatments varied significantly with respect to all the factors. We observed significant difference in the proliferative response of antler cells between two examined concentrations of foetal calf serum. In the high concentration, the responses were more intense and for some treatments even opposite to the ones in the low concentration. The same goes for primary versus passaged cultures. As for the sampling day, the cells sampled on the 15th day after antler casting proliferated the most intensively. In our experiments we did not observe any consistent effect of the treatments. Generally, testosterone stimulated or did not show any effect on the antler cell proliferation. In contrast, IGF-1 did not stimulate or even inhibited the antler cell proliferation. Antisteroidal treatments and estradiol showed no general trend. Unfortunately we could not perform hormonal experiments on the stem cells isolated from the mixed antler cell cultures, since we were unable to obtain sufficient amounts of positive cells or to expand their numbers while keeping the undifferentiated potential. Despite this fact, our findings suggest that sex steroids play an important role in stimulation of antler growth but their effect seems to be time- and antler-stage dependent. In addition, we could not confirm the mitogenic effect of IGF-1 reported by the previous *in vitro* studies [32–34]. Our results are in accordance with many physiological and behavioural *in vivo* studies and support the role of testosterone in the antler re-growth phase [13]. Furthermore, our results suggest that the primary cultures may better represent *in vivo* conditions and processes that occur in regenerating antlers.

In conclusion, we believe that testosterone might be the "antler growth stimulus", but since there are many factors influencing antler cell proliferation *in vitro* and antler regeneration/re-growth study is in its beginning, there is a need for further and more detailed experiments that could confirm this hypothesis.

4 Summary of Papers

The thesis consists of four papers. Each of them is presented in the following separate chapter.

Paper I
Effect of different factors on proliferation of antler cells, cultured *in vitro*. Erika Kužmová, Luděk Bartoš, Radim Kotrba, George A. Bubenik (2011) PLoS ONE 6(3): e18053. doi:10.1371/journal.pone.0018053

Paper II
Factors affecting the number of STRO-1+ stem cells derived from regenerating antler and pedicle cells of red and fallow deer. Erika Kužmová, Radim Kotrba, Hans J. Rolf, Luděk Bartoš, Günter K. Wiese, Jutta Schulz, George A. Bubenik (2011) Animal Production Science 51 (4) pp. S35-39.

Paper III
The effect of testosterone and IGF-1 on antler cell proliferation *in vitro*. Erika Kužmová, Luděk Bartoš, Radim Kotrba, Hans J. Rolf, George A. Bubenik. *Submitted to an international journal.*

Paper IV
Endocrine relationships between rank-related behavior and antler growth in deer with a focus on *in vivo* studies. Luděk Bartoš, George A. Bubenik, Erika Kužmová (2011) Frontiers in Bioscience. *In press.*

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PAPER I

Effect of Different Factors on Proliferation of Antler Cells, Cultured in Vitro

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Effect of Different Factors on Proliferation of Antler Cells, Cultured *In Vitro*

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Abstract

Antlers as a potential model for bone growth and development have become an object of rising interest. To elucidate processes explaining how antler growth is regulated, *in vitro* cultures have been established. However, until now, there has been no standard method to cultivate antler cells and *in vitro* results are often opposite to those reported *in vivo*. In addition, many factors which are often not taken into account under *in vitro* conditions may play an important role in the development of antler cells. In this study we investigated the effects of the antler growth stage, the male individuality, passaged versus primary cultures and the effect of foetal calf serum concentrations on proliferative potential of mixed antler cell cultures *in vitro*, derived from regenerating antlers of red deer males (*Cervus elaphus*). The proliferation potential of antler cells was measured by incorporation of ^{3}H thymidine. Our results demonstrate that there is no significant effect of the antler growth stage, whereas male individuality and all other examined factors significantly affected antler cell proliferation. Furthermore, our results suggest that primary cultures may better represent *in vivo* conditions and processes occurring in regenerating antlers. In conclusion, before all main factors affecting antler cell proliferation *in vitro* will be satisfactorily investigated, results of *in vitro* studies focused on hormonal regulation of antler growth should be taken with extreme caution.

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Introduction

As the only completely regenerating organ found in mammals, deer antlers evoke rising interest of many scientists. Antlers can be used as an interesting and easily accessible model for bone growth processes as well as mammalian regeneration [1–3]. On the other hand, despite decades of being studied, a lot is still unknown about the regulation of antler growth. Various authors carried out *in vivo* and *in vitro* experiments and in many cases the correlations between antler growth and various hormones or growth factors, testosterone and IGF-1 in particular, and their effect on antler growth, are contradictory [1,4–11]. As suggested earlier [8,11], this inconsistency may lie in factors associated with the *in vitro* environment. Indeed, recently an increasing interest is paid to the influence of cultivation factors which can affect the proliferation and differentiation potential of cell cultures in vitro. This shows up especially for mesenchymal stem cell cultures [12–21]. Mesenchymal stem cells (MSC) were lately isolated also from pedicles and regenerating antlers of fallow deer [3]. Recently we confirmed that considerable amounts, up to 38% of these cells can be isolated from the regenerating antler tips of fallow and red deer, even though the amount of isolated MSC varied greatly depending on culture conditions [22].

Throughout the literature, experiments using pedicle [4,5] or antler cells [9–11,23], cells from different stages of antler development and growth, cultivated either as primary cultures [23] or after two passages [4,5,9–11], grown in medium containing foetal calf serum (FCS) [4,5,9–11,23] or partially cultured in serum free conditions [4,5,9,10] have been reported. Despite all these differences, there was no attempt to study possible effects of these factors on growth and development of antler cells *in vitro*, although they all may be of high importance.

Another possible factor influencing the antler cells *in vitro* is the individuality of each animal, i.e. inter-individual differences among the cells from different animals. This is important, since inter-individual variation of antler growth and size plays a significant role in the social behaviour and reproductive success of the deer species [24,25]. Inter-individual differences are also an often-described feature of mesenchymal stem cells [13,20,26,27]. However, individuality has not been explicitly taken into account in any of the *in vitro* experiments on antler cells [4,5,9–11,23].

In the presented study we investigated the significance of factors affecting the proliferation potential of antler cells from three individual red deer males (*Cervus elaphus*). Samples were taken from the regenerating antler tip during the most rapid growth phase of antlers on the 30th and 60th day of the antlers re-growth after previous antlers were cast [2]. The cell proliferation was measured by incorporation of ^{3}H thymidine in primary cultures or in the second passage cultures and cultivated with 10% or 1% of FCS. We hypothesized, that inter-individual differences will show up in all culture conditions, identically in both sampling days, but may vary with changing passage and percentage of FCS.

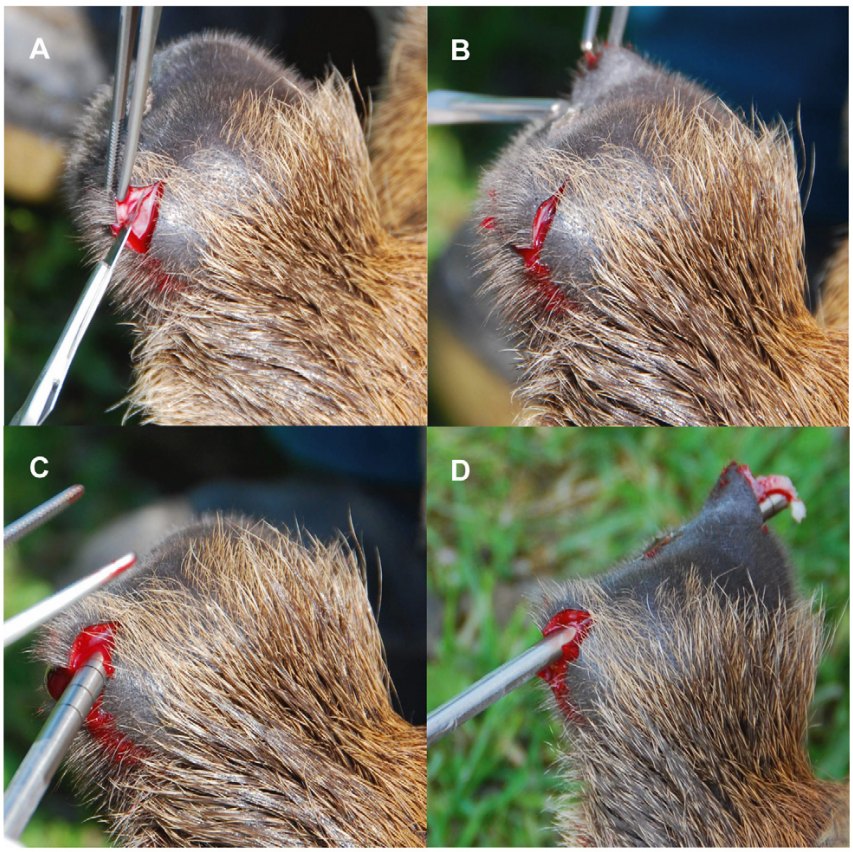

Figure 1. Tissue sampling. Example of tissue sampling from anesthetized animals using a sterile trephine punch.
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Materials and Methods

Antler tissue

All experiments were conducted under the approval of the Institute of Animal Science and Central Commission for Animal Welfare (Ministry of Agriculture of the Czech Republic) Committee (protocol code 26847/2006-17210).

Three three-year old farmed red deer males were fully immobilized with 30 ml intramuscularly injected Hellabrunn mixture [187.5 mg Xylazine (Bioveta, Prague, Czech Republic) +150 mg Ketamine (Bioveta, Prague, Czech Republic) in 1 ml, used 0,2 ml/10 kg of life weight] by a veterinarian in a crush. Subsequently the growing tips of regenerating antlers were superficially cleaned with a disinfection agent Spitaderm (Ecolab, 509-302056). Approximately 0.5 – 1.0 cm from the antler tip where the growth zone was reported [28,29] a biopsy was taken. This zone is considered as an abundant source of cells for *in vitro* studies [1,30]. The biopsies were performed on the 30^{th} and the 60^{th} day after the initiation of a new antler growth. The epidermis and the dermis were cut with a scalpel in a "V" shape and were diffracted to enable the underlying tissue for the biopsy. This was performed with a sterile trephine punch (Ø6 mm, Eickemeyer, 184905) (Fig. 1.). The obtained tissue was immediately put into a sterile tube containing "manipulation medium" DMEM/F12 containing 1% Insulin-Transferin-Selenium Supplements (ITS), 1% Antibiotic Antimycotic solution, 0,1% Gentamycin and 5% FCS (all reagents were from Gibco/Invitrogen, Prague, Czech Republic).

Cell isolation and culture conditions

The tissue was processed immediately (within 30 min.) after the biopsy. The cells were acquired by a combination of two methods as described by Sadighi et al. [9] and Faucheux et al. [23]. Briefly, the tissue was washed with Hanks Balanced Salt Solution containing 1% Antibiotic Antimycotic solution and 5% FCS. Specimens were mechanically minced into pieces approximately 0.5–1 mm^3 in size using a sterile scalpel, under aseptic conditions in a laminar flow hood, washed again and incubated in "standard medium" DMEM/F12 1:1 containing 1% Penstrep, 1% ITS and 0,1% Gentamycin with 200 U/ml Type II Collagenase (Gibco/Invitrogen, Prague, Czech Republic) for 4 hours at 37°C. Samples were continuously vortexed every 20 min. Obtained cells were immediately sieved and seeded into experiment as primary culture (60^{th} day after antler casting) or cultivated in the density of $4–5.10^4$ cells per cm^2 until reaching confluence and second passage (within 6–8 days) was seeded into the experiments (30^{th} and 60^{th} day after antler casting). In both cases, cells were seeded in 48-well plates (Nunc) at a density of 4.10^4 cells per well, followed by a 24-hour-cultivation in 1% FCS and by a 2×24-hour-cultivation in 1% or 10% FCS, all in a triplicate way. The cells were incubated at 37°C in 5% CO_2 and 95% air.

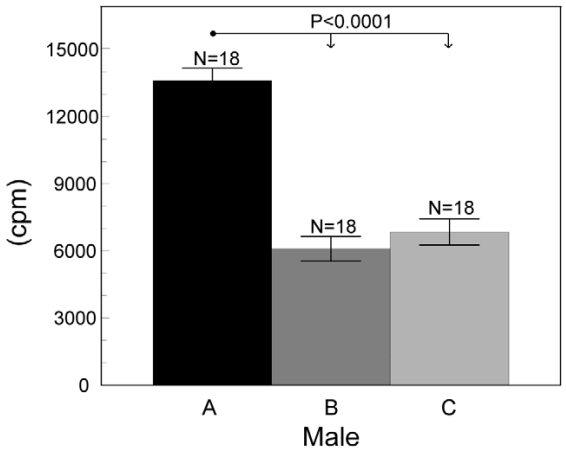

Figure 2. Effect of the individual males on the antler cell proliferation. Incorporation of ^{3}H thymidine in antler cells (least square means ± S.E.) according to the individual males (A, B, C). All other factors were statistically eliminated.
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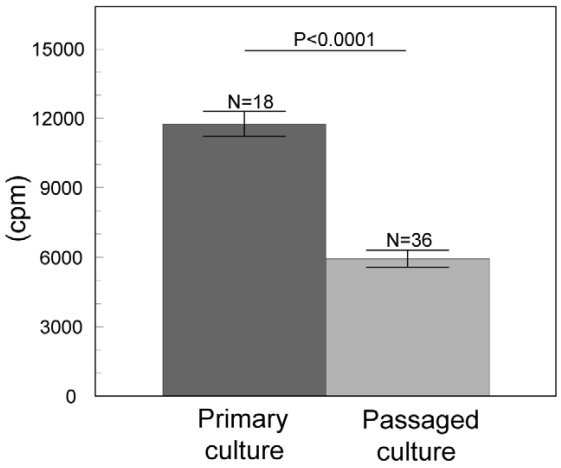

Figure 3. Effect of the passage on the antler cell proliferation. Incorporation of ^{3}H thymidine in antler cells (least square means ± S.E.) according to the passage (primary culture, passaged culture – 2nd passage). All other factors were statistically eliminated.
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Cell proliferation essay

To determine the cell proliferation potential, 16 hours before the termination of incubation ^{3}H thymidine (Methyl-^{3}H thymidine, s. a. 6–7 Ci/mmol, ICN, USA) was added in the final concentration of 1 μCi/ml into each well. The DNA synthesis was measured by incorporation of ^{3}H thymidine using the technique of TCA precipitation and liquid scintillation counting as described in Vacková et al. [31].

Statistics

Associations between antler cells proliferation, two antler growth stages (30, N = 12 and 60, N = 36, days after the antler casting), individual males (A, N = 18; B, N = 18 and C, N = 18), the passage (primary culture, N = 18 and passaged cells, N = 36) and the percentage of FCS (1%, N = 27 and 10%, N = 27) were tested using multivariate General Linear Mixed Model (GLMM) with incorporation of ^{3}H thymidine as the dependent variable and the variables described above as fixed effects. To account for the repeated measures on the same individuals, all analyses were performed using mixed model analysis with individual deer in an interaction with the passage as a random factor, using PROC MIXED (SAS, version 9.1). The significance of each fixed effect in the mixed GLMM was assessed by the F-test, on sequential dropping of the least significant effect, starting with a full model. In unbalanced designs with more than one effect, the arithmetic mean for a group may not accurately reflect a response for that group, since it does not take other effects into account. Therefore, we used least-squares-means (LSMEANs) instead. LSMEANs are, in effect, within-group means appropriately adjusted for the other effects in the model. LSMEANs were computed for each class and differences between classes were tested by t-test. For multiple comparisons we used the Tukey-Kramer adjustment.

Results

Proliferation of growing antler cells depended on all investigated factors (such as male individuality, passage and percentage of FCS) but not on the stage of antler growth. The final GLMM model contained fixed effects of the male individuality ($F_{(2,\ 46)} = 56.11$, $P<0.0001$ Fig. 2), passage ($F_{(1,\ 46)} = 80.53$, $P<0.0001$ Fig. 3), percentage of FCS ($F_{(1,\ 46)} = 210.65$, $P<0.0001$ Fig. 4) and an interaction between individual males and cell passage ($F_{(2,\ 46)} = 101.37$, $P<0.0001$ Fig. 5). The proliferation of antler cells was highly affected by male individuality. As predicted, the intensity of proliferation of particular individuals was identical between the two antler growth stages, since no significant effect of antler growth stage was confirmed. Higher percentage of FCS (10%) emphasized the inter-individual differences among the males apparent in the 1% FCS, while passage changed the proportion of the proliferative intensity among the males (Fig.4). Moreover cells of particular individuals cultivated as a primary culture, without passaging, reacted with significantly higher intensity than cells after passage. Not surprisingly 10% of FCS stimulated cell proliferation more than 1% of FCS.

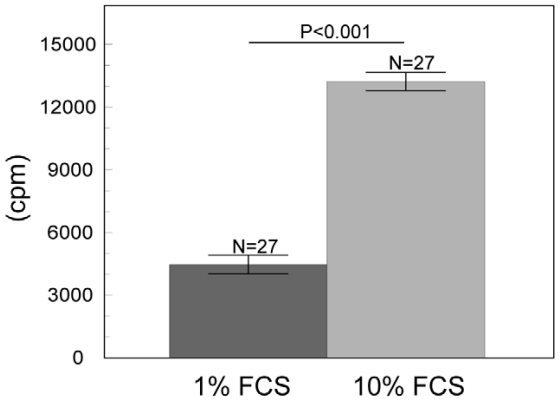

Figure 4. Effect of the FCS on the antler cell proliferation. Incorporation of ^{3}H thymidine in antler cells (least square means ± S.E.) according to FCS percentage. All other factors were statistically eliminated.
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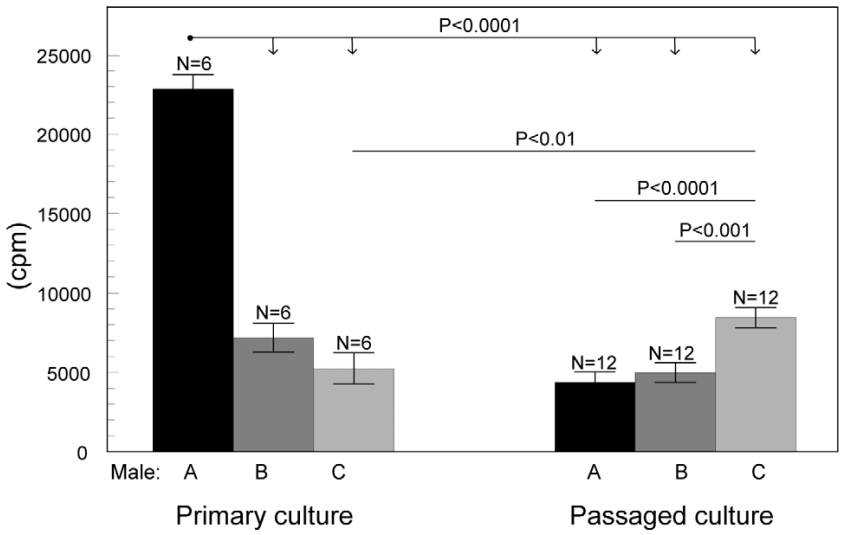

Figure 5. Effect of the interaction between individual males and passage on the antler cell proliferation. Incorporation of ^{3}H thymidine in antler cells (least square means ± S.E.) - the interaction between individual males (A, B, C) and passage (primary culture, passaged culture – 2nd passage). All other factors were statistically eliminated.
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Discussion

In agreement with our predictions, the results clearly show that the factors such as 1) male individuality, 2) whether the antler cells were passaged or not and 3) concentration of FCS in the cultivation medium significantly affected antler cell proliferation *in vitro*. The only tested factor, which did not influence the antler cell proliferation, was the stage of antler growth.

Our work differs from the previously published works by sampling on both the 30th and the 60th day after antler casting, from the same individual. In this way we obtained and compared cells twice during the antler growth phase. However the time interval between the two growth stages on the 30th and 60th day was probably not sufficient to demonstrate any significant differences and samplings from earlier stages would be needed to point out potential differences in the proliferation intensity of antler cells.

Over the last years, a stem cell based origin of antlers was discussed and confirmed [32–36] and stem cells were found and isolated from regenerating antlers [3]. These MSC positive to surface antigen STRO-1 were shown by Rolf et al. [3] to differentiate into the "mesenchymal stem cell golden standard" - osteogenic, adipogenic and chondrogenic lineages. MSC are of great biomedical promise and a vast research interest is dedicated to their biology [37]. Recently we have shown that considerable amounts of MSC (up to 38%) can be isolated from mixed antler cell cultures [22]. This allows us to compare some of the MSC culture characteristics to our antler cell cultures.

We found a highly significant effect of male individuality on proliferation potential of antler cells. Similarly, a great inter-individual variability has been reported for ovine mesenchymal stem cell colonies [13] and for rabbits in the proliferative behaviour of the bone-marrow mesenchymal progenitor cells [20]. Ciapetia et al. [26] reported highly variable osteogenic potential in femur-derived human MSC among patients, unrelated to sex or age. In another study, Riekstina et al. [27] found very high inter-individual proliferation variability in skin-derived mesenchymal stem cell and their response to fibroblast growth factor-2, which after 3 days in culture overrode the effect of the growth factor and a generalized estimate of its effect was not possible.

In the present study, the rate of antler cell proliferation was significantly higher in 10% FCS than in 1% FCS in both primary and passaged culture. Such a result is not particularly surprising considering that cells in general proliferate more intensive in 10% FCS than in 1% FCS [19,38]. Berg et al. [39] reported that 81.9% of undifferentiated antlerogenic periosteum cells proliferate in 10% FCS whereas just 1.4% of cells cultivated in 0.5% FCS, which is similar to our observation.

Using 10% FCS may also lead to a reduced or changed expression of biochemical markers. Pradel et al. [19] did not find any significant effect of 10% FCS on the human osteoblast-like cells morphology between primary and second passage culture. On the other hand Pochampally et al. [40] reported, that the human mesenchymal stem cells (hMSC) cultivated in 10% FCS differentiate and change their superficial expression markers more quickly, while cells cultivated without serum express the markers of undifferentiated cells much longer. Yokoyama et al. [41] demonstrated that components of FCS could stimulate hMSC differentiation to chondrocytes while a lower concentration could decrease this differentiation. This is in contrast to Price et al. [42], who stated that unlike mesenchymal cells from a developing limb, the antler cells in the culture spread out, form monolayers and do not initiate chondrogenesis. Nevertheless, previously mentioned studies have indicated that independently of performing the experiments in serum free conditions, the precultivation of antler cells in 10% FCS [9–11] may cause the cells to react differently from cells of primary culture or cells *in vivo/in situ*. This could explain the differences among results of various studies of hormonal and grow factors influence on antler cell proliferation [1,4,5,10,11]. Experiments using FCS during precultivation should therefore be interpreted with caution and it seems more appropriate to simulate *in vivo* conditions by primary cultures with

only shorter exposure to FCS, as it was done by Faucheux et al. [23].

On the other hand, there are interesting indications by Patel et al. [17] on pulpal tissue, where the expression of markers regarded as being indicative of odontoblasts are considerably under-represented in primary culture compared to pulpal tissue. Hence cells immediately isolated and passaged no longer accurately represent intact pulpal tissue. They explain this due to either loss of specific cell populations as a result of the dissociation and adhesion processes or transcriptional changes within the isolated cells due to altered environmental conditions. In the same study continued cultures demonstrated more pronounced differences, which may in their opinion represent cellular adaptation and/or selection for a particular cell population with enhanced ability to thrive on tissue culture plastic. Indeed, in agreement with Patel's' study, Uchida et al. [16] showed that primary culture and second passage of rat mesenchymal bone marrow cells differ radically in the proportion of three detected cell populations.

As indicated above, during passaging, which is often performed to obtain sufficient numbers of cells, the cells change their morphology, capability to multiply and differentiate, and their gene expression changes dramatically [14–19]. A variation of the gene expression during passaging was confirmed also in cell lines [43] and the authors warn that even comparisons of analyses of cell line cultures carrying the same name may be dangerous.

In conclusion most *in vitro* hormonal and growth factor experiments with cultivated pedicle and antler cells have so far been performed after two passages [4,5,9–11]. Li et al. [5] stated that the reaction of such cells might represent the *in vivo* situation. This however is notably in contrast to recent literature and our results which show, that primary culture without any passaging and long term FCS treatment may be more related to the *in vivo* conditions. We suggest, that before all possible main factors affecting antler cells proliferation *in vitro* will be satisfactorily investigated, results of *in vitro* studies focused on hormonal regulation of antler growth [1,4,5,9–11] should be taken with increased caution.

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Author Contributions

Conceived and designed the experiments: EK LB. Performed the experiments: EK. Analyzed the data: EK LB. Contributed reagents/materials/analysis tools: RK. Wrote the paper: EK LB RK GAB.

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PAPER II

Factors Affecting the Number of STRO-1+ Stem Cells Derived from Regenerating Antler and Pedicle Cells of Red and Fallow Deer

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Factors affecting the number of STRO-1+ stem cells derived from regenerating antler and pedicle cells of red and fallow deer.

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Abstract:
Mesenchymal stem cells positive to surface antigen STRO-1 were isolated from regenerating antlers of red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) using a magnetic cell separation method. In this study we analysed factors potentially affecting the number of STRO-1+ cells in the cell cultures. With regard to the STRO-1 antigen, we evaluated data from 188 MACS® separation procedures of cell cultures cultivated in DMEM and 10% foetal calf serum of four fallow deer males (130 procedures) and four red deer males (58 procedures). The analysed factors were the sampling site of the antler or the pedicle, cell passage and type of the cell culture (mixed or STRO-1 negative cell cultures). The percentage of obtained STRO-1+ cells varied greatly from 0.4% to 38.9% for fallow deer and from 1.8% to 16.5% for red deer. We have not found any significant influence of the sampling site. The passage and the type of culture were significant factors for both fallow and red deer cells. The highest numbers of STRO-1+ cells were obtained from the second passage from both fallow and red deer cell cultures (24.6% ± 14.37, 5.5% ± 3.03 respectively). Our experiments revealed that we can maximize the number of STRO-1+ cells in the cultures by manipulating the cultivation factors.

Keywords: magnetic cell separation, cell culture passage, culture conditions, STRO-1+ stem cells

1 Introduction

Stem cell based origin of deer antlers has been discussed over the last decade and has been indirectly confirmed by various transplantation and deletion experiments of the antlerogenic and pedicle periosteum (Li and Suttie 2001, 2006, Mount et al. 2006, Li et al. 2007, Kierdorf et al. 2007, 2009). Rolf et al. (2006, 2008) described

the localization, isolated and characterized stem cells from pedicles and regenerating antlers of fallow deer (Dama dama) using the STRO-1 mesenchymal stem cell surface marker (Dennis et al. 2002). A creation of a new stem cell line from antlerogenic cells and successful xenoimplantation of these cells followed (Cegielski et al. 2008, 2010).

Each year, the pedicle periosteum gives rise to the antlers (Li et al. 2007). As shown by various authors, antlerogenic and pedicle periosteum cells express markers of undifferentiated multipotential cells (e.g. Oct4, Nanog) and mixed cultures of these cells could differentiate into osteocytes, chondrocytes and adipocytes under appropriate culture conditions (Li and Suttie 2006, Mount et al. 2006, Berg et al. 2007). We assume, that the STRO-1 positive (STRO-1+) cells, as a part of the antlerogenic tissue, could be one of the most important stem cell populations which give rise to chondroprogenitors and osteoprogenitors. STRO-1+ cell cultures differentiate in vitro along osteogenic, chondrogenic and with antler re-growth unrelated adipogenic lineages. Identification of particular cell types participating at the amazingly rapid antler re-growth might help to elucidate mechanisms behind this unique mammalian phenomenon frequently discussed as a suitable biomedical model for bone regeneration. Furthermore, such cells could provide an appropriate culture model e.g. to study hormonal influences during antler regeneration. A common problem of mesenchymal stem cells is the isolation of sufficient quantities of cells and their subsequent expansion. The culture conditions and cell passaging are crucial for maintaining their undifferentiated potential. Another problem with STRO-1+ cells is their rather wide range of incidence in the cell cultures (Stewart et al. 1999). Stewart et al. (1999) isolated 10-50% from murine bone marrow-derived cell line and 2-80% from adult human bone marrow stromal cells. In their opinion, the exact proportion of STRO-1+ cells was remarkably donor-dependent for the human cell cultures. Moreover they reported that STRO-1 negative (STRO-1-) fractions can give a rise to STRO-1+ cells.

In the present study we isolated mesenchymal stem cells positive to superficial antigen STRO-1 from regenerating red deer antlers by the same method as applied previously in fallow deer (Rolf et al. 2008). These cells should have served us as a model to examine the influence of hormones, particularly steroids and insulin like growth factor 1 (IGF-1), whose function is controversial in antler research (Bartoš et al. 2009). Additionally they could also support the prediction of inter-individual differences of antler growth, as indicated by our previous study on mixed antler cell cultures (Kužmová et al. 2011). For both fallow and red deer, the number of obtained STRO-1+ cells in the antler and pedicle cell cultures varied greatly. Due to initially planned hormonal experiments we wanted to avoid STRO-1+ cell cultures expansions by supplementation with growth factors. Besides, by expansion in standard cultivation conditions (DMEM and 10% FCS) or even in a special serum free expansion medium (Miltiney Biotec, Germany) the STRO-1+ cells lost their positivity rapidly (unpublished observation). For it was difficult to expand the STRO-1+ cells after separation, it became essential to determine the factors influencing the abundance of STRO-1+ cells in the cell cultures prior to the MACS® separation. In this study we present a detailed analysis of the factors sampling site, cell passage

and type of cell culture, which possibly affected the percentage of STRO-1+ cells in the fallow deer and red deer cell cultures.

2 Materials and Methods

The tissue was collected from growing antlers 15 to 90 days after antler casting from four anesthetized red deer males and four fallow deer males by bioptic punch 1 and 2 cm below the growing antler tip corresponding to the antler growth and cartilaginous zone (Matich et al. 2003). To obtain additional samples from antler bone, antler periosteum and pedicle periosteum the four adult fallow deer were slaughtered and sampled according to Rolf et al. (2008). The age of the deer males was between two and six years. The obtained samples were mechanically minced into pieces (approx. 0.5 - 1 mm3). The tissue pieces of red deer were incubated for four hours at 37°C in Dulbecco's Minimal Eagle Medium (DMEM) supplemented with antibiotics and 200 U/mL Type II Collagenase (Gibco/Invitrogen, Czech Republic) and vortexed every 20 min. (Kužmová et al. 2011). The cells of fallow deer were let grown out of cultured tissue pieces. All cells and tissue pieces were cultured in DMEM supplemented with 10% FCS under standardized conditions (37°C and 5% CO2). Because the cultivation density was not specified in some of the primary tissue cultures, it could not be analyzed as one of the factors.

The mixed cell cultures were passaged after reaching subconfluence up to 2nd passage for red deer and up to 7th passage for fallow deer. Mixed cell cultures were labeled with a primary antibody, surface antigen STRO-1 (R&D Systems, Germany), coupled with secondary antibody IgG-MicroBeads (Miltenyi Biotec, Germany) and separated with MACS® (Miltenyi Biotec, Germany) according to the manufacturer's protocols. The cells were counted before and after MACS® separation using a CASY cell counter. After the MACS® separation the STRO-1- fraction was further cultivated until the cells reached subconfluence and then separated to examine the number of STRO-1+ cells (these cultures will be referred to as STRO-1- cultures and their passage numeration continues from the primary cultures). To ensure that no STRO-1+ cells remained in the negative fractions, the procedure was repeated immediately after the first separation in few cases. Two of the STRO-1- fractions have been further cultivated in DMEM and 10% FCS until they reached subconfluence and separated again up to six cultivation and separation procedures (always cultivating only the negative fraction). These multiple-times cultivated and separated STRO-1- cell cultures were not statistically evaluated and are discussed separately in this study. All MACS® separations were performed in the same laboratory as part of a long term study on antler stem cells. The data from fallow and red deer were statistically analysed separately. The procedures of 188 MACS® separation were analysed, 130 for fallow deer and 58 for red deer. For the statistical analysis we used the General Linear Mixed Model (GLMM) using the PROC MIXED procedure (SAS V9.0) with least-square-means (LSMEANs) and the Tukey-Kramer adjustment for multiple comparisons. To account for the repeated measures on the same individuals, all analyses were performed with individual males as a random

factor. The dependent variable was the percentage of obtained STRO-1+ cells (calculated from the total number of cells after separation) and was analysed in relation to the following factors: (a) the sampling site (antler growth zone and antler cartilaginous zone for fallow and red deer; antler bone, antler periosteum and pedicle periosteum for fallow deer), (b) cell passage and (c) type of cell culture (mixed cell culture and STRO-1- cell culture).

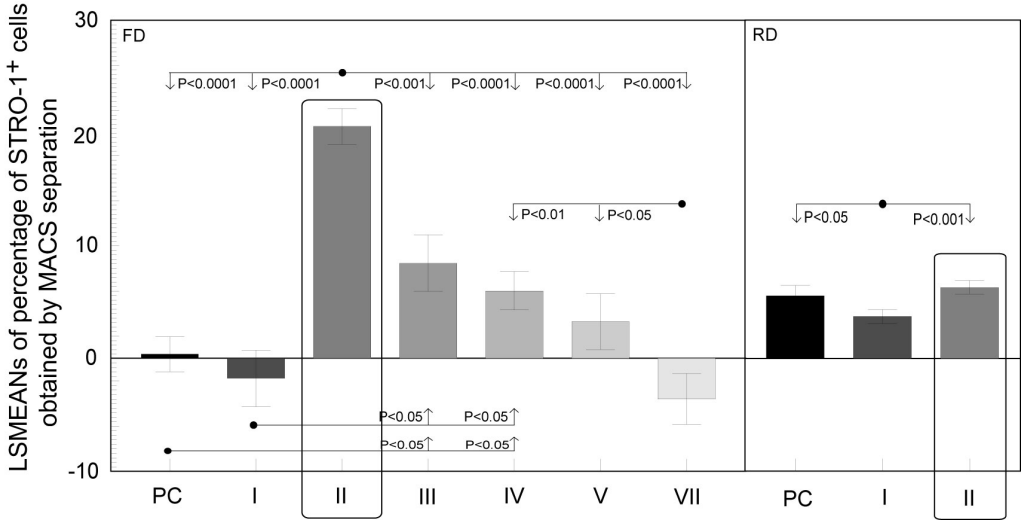

Figure 1: Effect of the passage (primary culture PC, passage I. - VII.) on the STRO-1+ quantities by fallow (FD) and red deer (RD). The 6^{th} passage of the fallow deer cell cultures was not separated.

3 Results

We examined the influence of particular sampling site and cultivation factors on the percentage of STRO-1+ cells in the cell cultures. The percentage of obtained STRO-1+ cells varied between 0.4% and 38.9% for fallow deer and between 1.8% and 16.5% for red deer.

We did not detect any significant influence of the cell sampling site of the antler or pedicle on the percentage of obtained STRO-1+ cells.

For both red and fallow deer cells the passage was a highly significant factor (red deer: $F_{(2,51)} = 8.41,\ P < 0.001$, fallow deer: $F_{(6,122)} = 33.6,\ P < 0.0001$). In general, the highest percentage of STRO-1+ cells was obtained from the second passage, both in fallow deer (24.6% ± 14.37) and red deer (5.5% ± 3.03) as shown in Figure 1.

The type of culture was also a significant factor (red deer: $F_{(1,51)} = 4.44,\ P < 0.05$, fallow deer: $F_{(1,122)} = 18.75,\ P < 0.0001$), but the results differed between fallow and red deer. While the percentage of obtained STRO-1+ cells from a mixed culture was significantly higher compared to the STRO-1- culture of fallow deer, it was the opposite for red deer cells (Fig. 2).

By immediate repetition of the MACS® separation procedure on the STRO-1- fraction, we hardly detected any STRO-1+ cells remaining in the STRO-1- fractions. However, after subsequent cultivation we isolated STRO-1+ cells from multiple-times cultivated and sorted STRO-1- cell cultures.

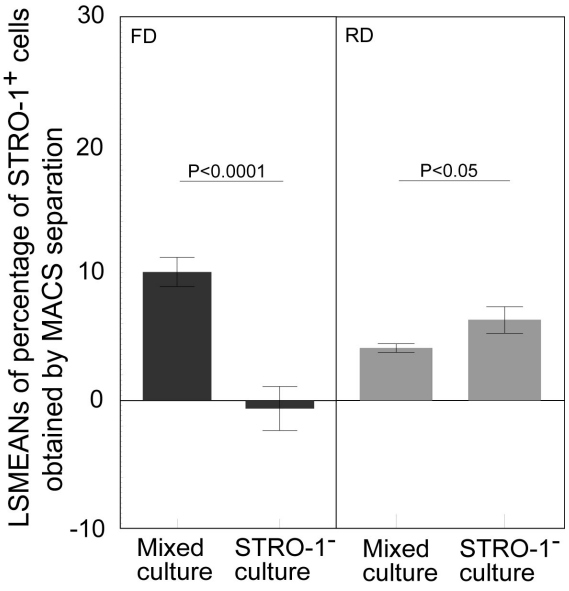

Figure 2: Effect of the type of culture on the STRO-1+ quantities by fallow (FD) and red deer (RD).

4 Discussion

This study focused on factors which could influence the yields of STRO-1+ cells in cell cultures derived from regenerating antlers and pedicle of fallow deer and red deer. The amount of obtained STRO-1+ cells was highly variable as it was also described in Stewart et al. (1999). We determined that the number of passages and type of cell culture were both significant factors. On the other hand, we found no significant influence of the sampling site of antlers or pedicles. It is hence probable that the cultivation procedure affected the yields of STRO-1+ positive cells up to the level that overrides the anticipated effect of sampling site, particularly the pedicle periosteum, which is supposed to be the initiation tissue for antler re-growth (Li et al. 2007).

In our experiments, we have isolated STRO-1+ cells not only from subsequently cultivated STRO-1- fractions as previously reported by Stewart et al. (1999), but also from multiple-times cultivated and sorted STRO-1- cell cultures. As STRO-1+ cells were not detected in STRO-1- cell fractions right after the separation, the STRO-1+ cells isolated from subsequently cultivated negative cultures had to arise from the STRO-1- cells. At this stage we can only speculate about the explanation. First, negative cultures may contain earlier precursor cells which after some time

of cultivation become STRO-1+. Second, as a result of the in vitro cultivation, 10% of FCS and passaging, differentiated cells might de-differentiate and become STRO-1+. Such de-differentiation is typical especially for chondrocytes cultivated as monolayers (Barbero et al. 2003). Third, the used method was not sensitive enough to separate cells which possess only small number of STRO-1 surface proteins, and subsequently, their number increased during further cultivation.

The second passage of the cell cultures cultivated in DMEM and 10% FCS seemed to be the most suitable for the isolation of the greatest numbers of STRO-1+ cells. This could be explained by the de-differentiation of particular cells in the cultures as well as by the presence of cells in various stages of expression of STRO-1 marker, their continuously changing multilineage potential and ongoing differentiation (Yu et al. 2010, Stewart et al. 1999, Barbero et al. 2003). Moreover, Simmons and Torok-Storb (1991) found a comparable pattern of a significant increase in the proportion of STRO-1+ cells after two weeks of cultivation, followed by a progressive decline. They concluded that it could be a culture epiphenomenon unrelated to normal in vivo conditions, or it occurred due to the maturation of stromal precursors into more differentiated stromal cell types as discussed above.

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PAPER III

The Effect of Testosterone and IGF-1 on Antler Cell Proliferation in Vitro.

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The effect of testosterone and IGF-1 on antler cell proliferation *in vitro*.

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Abstract:

Sex steroids are required for antler development and have a well established role in the timing of the annual antler cycle. However, there is no unified opinion whether the "antler growth stimulating hormone" may be testosterone or IGF-1 and the discrepancy is mainly between the *in vivo* and *in vitro* reports.

In this study we examined the proliferative response of mixed antler cell cultures to sex steroids and IGF-1 treatments *in vitro*. Cells were derived from regenerating antlers of red deer males (*Cervus elaphus*) on the 15th, 30th and 60th day after antler casting. The proliferation potential of antler cells was measured by incorporation of ^{3}H-thymidine. We determined that testosterone stimulated antler cell proliferation or had no effect. On the other hand, IGF-1 did not stimulate the proliferation of antler cells in any of the experiments performed in this study; it either inhibited the proliferation, or had no effect. In a few cases IGF-1 inhibited the stimulating effect of testosterone. Antisteroidal treatments and estradiol showed no general trend. The effect of all treatments and intensity of the antler cell proliferation varied depending on the duration of the experiment, the day on which the tissue sampling was performed (antler growth stage) and the concentration of used foetal calf serum (FCS). Compared to other sampling days, the cells from the 15th day exhibited highest proliferation rates. These findings suggest that sex steroids play an important role in the stimulation of antler growth but their effect is time- and antler-stage dependent. We could not confirm the stimulating effect of IGF-1 reported in previous *in vitro* studies.

Keywords: antler cells, proliferation, testosterone, IGF-1, estradiol, antler growth stage

1 Introduction

Sex steroids have a crucial role in bone development [1]. As antlers are the only mammalian bony organ that fully regularly regenerates, they present a unique model to study the role of sex steroid hormones in the developing bone.

When young deer males approach puberty, pedicles develop and the primary antlers grow, mineralize and are cast [2]. Henceforward, the following cycle repeats annually: after the antlers have been cast in the spring, the re-growth of new pair starts immediately, reaching the fastest growth of up to 1 cm per day in red deer in late spring [3]. During summer antlers mineralize and velvet shedding occurs. The rutting season follows and the antlers persist until next springtime. It is generally accepted that sex steroids are required for pedicle- and primary antler development and are most important for the timing of annual events in the antler cycle [4]. While increasing levels of testosterone cause antler mineralization and velvet shedding, their rapid decline below distinct threshold values during springtime cause the antler casting [5, 6]. During the antler re-growth, the systemic levels of testosterone are at their minimum and hence sex steroid hormones were assumed to play only a minor role in the antler growth stimulation [4, 7, 8]. Although, both androgen and estrogen receptors were localized in growing antlers at the time when circulating concentrations of sex steroids are generally low [4, 9, 10].

Suttie et al.'s [11] hypothesis that IGF-1 is the main antler stimulating hormone was supposed to replace an earlier theory that the antler-stimulating hormones are either androgens or their derivatives [5, 12–15]. Recently, we presented historical and recent views on these issues [16]. In particular, we analyze the arguments in favor and against the role of testosterone and IGF-1 in antler growth and present a comparison of the results obtained across some deer species. We concluded that testosterone and not IGF-1 is the main antler stimulating and regulating hormone in *in vivo* studies. Contrary to that, meta-analysis of the *in vitro* studies revealed inconsistency of the results reaching over 80% of total variation across studies due to heterogenity for testosterone and nearly 100% of that for IGF-1 [16].

Similarly to the *in vitro* studies favoring testosterone [16], most of the *in vitro* studies supporting the role of IGF-1 were performed on cells sampled 60 days after antler casting [17, 18] or the precise date of the sampling is not given [4, 19, 20] or experiments were performed on the developing pedicle and primary antler tissue [2, 21]. Moreover, it was suggested by Bubenik et al. [22] that the effects of testosterone in growing antler tissue might be mediated via local aromatization to estradiol as it generally occurs in many other bone tissues [23, 24]. However, the effects of estradiol on the proliferation of antler cells derived from the growing antler tips *in vitro* have not been examined yet.

In this study we performed *in vitro* experiments on population of antler cells derived from regenerating antlers of adult red deer males. The investigations were focussed on the proliferative effect of the sex steroids testosterone and 17β-estradiol (estradiol) and the insulin-like growth factor-1 (IGF-1). They were tested alone or together with antiandrogens cyproterone acetate (CA), flutamide and antiestrogen ICI 182,780. Furthermore, we compared cells from the same animals derived at

three different antler growth stages, in various experimental designs and with different concentrations of foetal calf serum (FCS). We hypothesized that testosterone as well as estradiol will stimulate the proliferation of antler cells and that the used antisteroids will block their effect. We also predicted that the length of the experiment and the stage of the tissue growth will have a significant influence on the proliferative response.

2 Materials and Methods

2.1 Method of Collection of Antler Tissue

Three two-year and five three-year old farmed red deer males (*Cervus elaphus*) were anaesthetized, the growing antler tips were superficially cleaned with a disinfectant and a biopsies were taken approximately 0.5-1 cm below the antler tip as described in our previous study [25]. Samplings were performed on the 15th, 30th and 60th day after the previous antlers had been cast from the two-year old males and on the 30th and 60th day from the three-year old males. The obtained tissue was immediately put into a sterile tube containing "manipulation medium" DMEM/F12 containing 1% Insulin-Transferin-Selenium Supplements (ITS), 1% Antibiotic Antimycotic solution, 0,1% Gentamycin and 5% FCS (all reagents were from Gibco/Invitrogen, Prague, Czech Republic). The samples were brought into the laboratory within 30 minutes of collection.

2.2 Cell Isolation and Culture Conditions

The cells were acquired by the method described previously [25]. Briefly, the tissue was washed with Hanks Balanced Salt Solution containing 1% Antibiotic Antimycotic solution and 5% FCS, diced into approximately 0.5-1mm^3 pieces, washed again and incubated in "standard medium" DMEM/F12 containing 1% Penstrep, 1% ITS and 0,1% Gentamycin with 200U/ml Type II Collagenase (Gibco/Invitrogen, Prague, Czech Republic) for 4 hours at 37°C in 5% CO_2 and 95% air. Samples were vortexed continuously every 20 min. Obtained cells were sieved and used in four kinds of hormonal experiments using the following concentrations of the treatments: testosterone (T) 1 nM, 10 nM; insulin like growth factor-1 IGF-1 (6nM IGF-1) 6.5 nM and (13nM IGF-1) 13 nM; combinations of testosterone and IGF-1; 17β-estradiol (E) 1 nM, 10 nM as well as Cyproterone acetate (CA) 100 nM, Flutamide (F) 100 nM and antiestrogen ICI 182,780 (ICI) 100 nM in various combinations. The control (C) samples was always underwent the same procedure, but without any hormonal treatment.

2.3 Methodical Perspectives

Our experiments were designed to repeat the experiments of Sadighi et al. [18] with slight differences specified in Table 1. To eliminate the possible factors of the

precultivation procedure, experiments 3 and 4 were performed without precultivation. The proliferation intensity might be low under such limited cultivation time, therefore parallel experiments were performed in 1% and 10% FCS. To examine the mechanisms of sex steroid stimulation we used the highly efficient steroidal receptor blockers, antiandrogens CA and Flutamide [26, 27] and antiestrogen ICI which were added to the cultures always 1h before the other hormonal treatments were added.

2.4 Experimental Designs

Cells from the three-year old deer males were precultivated in Petri dishes (Nunc) at densities of approximately $4 \cdot 10^4$ cells/cm^2 until reaching confluence and the cells from the 2nd passage (within 6-8 days) were seeded into the experiments at density of $2 \cdot 10^4$ cells/cm^2, using the 48-well plates (Nunc) in triplicate way. The cells were cultivated for 24 h in "standard medium" containing antibiotics and supplemented with 1% FCS. Afterwards hormonal supplements were added.

Experiment 1: The cells were treated with hormonal supplements for another 2x24 h and subsequently the experiment was terminated. Used treatment combinations and concentrations were C, 6nM IGF-1, 13nM IGF-1, 1nM T, 10nM T, 10nM T/6nM IGF-1, 10nM T/13nM IGF-1, 1nM E, 10nM E.

Experiment 2: The cells were treated with hormonal supplements for another 6x24 h and subsequently the experiment was terminated. Used treatments were the same as in experiment 1.

Cells from the two-year old deer males were immediately seeded into experiments as primary culture, without any precultivation. The cells were cultivated at density of $4 \cdot 10^4$ cells/cm^2 using the 24-well plates (Nunc) in triplicate or quadruplicate way for 24h in "standard medium" containing antibiotics and supplemented with 1% FCS. Afterwards hormonal supplements were added.

Experiment 3: The cells were cultivated for another 24h with 1% FCS and hormonal treatments C, 6nM IGF-1,13nM IGF-1 (only for the cells from the 60th day), 1nM T, 10nM T, 1nM T/6nM IGF-1, 10nM T/6nM IGF-1, CA, 10nM T/CA, 10nM T/CA/6nM IGF-1, F, 10nM T/F, 10nM T/F/6nM IGF-1, 10nM E, ICI, 10nM E/ICI.

Experiment 4: Cells were cultivated for another 24h with 10% FCS and all hormonal treatments used in the Experiment 3 were also applied.

Since we had not obtained sufficient amounts of cells by the described precultivation-free procedure, the following treatments were not performed: 13nM IGF-1, 10nM E, ICI, 10nM E/ICI in experiments 3 and 4 on cells from 15th day, and 13nM IGF-1 also for cells from 30th day.

The medium, FCS and all hormonal and culture supplements were changed every day. After termination of each experiment, the intensity of cell proliferation was measured.

Table 1: Comparison of the experimental design of the work by Sadighi et al. [18] and our experiments.

	Sadighi et al. 2001	Exp. 1,2	Exp. 3, 4
Differences in the methodology:			
Number of individuals	?	5	3
Days after antler casting	60	30, 60	15, 30, 60
Collagenase (h)	24 h	4 h	4 h (afterwards set into exp.)
^{3}H thymidine	85 Ci/mmol	6-7 Ci/mmol	6-7 Ci/mmol
^{3}H thymidine used conc.	2.5 uCi/ml	1 uCi/ml	1 uCi/ml
Lab. plastic used	24-well dish	48-well dish	24-well dish
Differences in the precultivation process:			
Passages before experiment	2	2	no
FCS during precultivation	10%	10%	no
Primary cult. dens. c/cm^2	$2 \cdot 10^4$ c/cm^2	unknown	no
1st passage cell dens. c/cm^2	$2 \cdot 10^4$ c/cm^2	$4 \cdot 10^4$ c/cm^2	no
2nd passage	frozen and then set into exp.	set into exp.	no
Experimental design:			
Length of the experiment	4x24h	3x24h (E1) 7x24 h (E2)	2x24 h
Experiment cell density	$2 \cdot 10^4$ c/ml	$2 \cdot 10^4$ c/cm^2	$4 \cdot 10^4$ c/cm^2
IGF-1 concentration	10nM	6nM, 13nM	6nM, 13nM
FCS during experiment (h)	2x24h 10% 2x24h serum free	1x24h 10% 2x24h 1%(E1) 6x24h 1%(E2)	1x24h 1% 1x24h 1% (E3) 1x24h 10% (E4)
Value of n per treatment (used for statistics and graphs)	3	n≥15	n≥9

2.5 Cell proliferation Essay

To determine the cell proliferation potential of the antler cells, 16 hours before the termination of each experiment ^{3}H thymidine (Methyl-^{3}H thymidine, s. a. 6-7 Ci/mmol, ICN, USA) was added in the final concentration of 1μCi/ml into each well. The DNA synthesis was measured by incorporation of ^{3}H thymidine using the technique of TCA precipitation and liquid scintillation counting as described previously [25].

2.6 Statistics

Associations between antler cells proliferation were tested using multivariate General Linear Mixed Model (GLMM) with number of cells (expressed in the form of incorporation of ^{3}H thymidine) as the dependent variable and fixed effects specified below for the Experiments 1 to 4. To account for the repeated measures on the same individuals, all analyses were performed using mixed model analysis with individual deer as a random factor, using PROC MIXED (SAS, version 9.1). The significance of each fixed effect in the mixed GLMM was assessed by the F-test, on sequential dropping of the least significant effect, starting with a full model. In unbalanced designs with more than one effect, the arithmetic mean for a group may not accurately reflect a response for that group, since it does not take other effects into account. Therefore, we used least-squares-means (LSMEANs) instead. LSMEANs are, in effect, within-group means appropriately adjusted for the other effects in the model. LSMEANs were computed for each class and differences between classes were tested by t-test. For multiple comparisons we used the Tukey-Kramer adjustment. Hormonal treatments were analyzed in two steps: First, for Experiments 1 and 2 we analyzed hormonal addition of 6nM IGF-1, 13nM IGF-1, 1nM T, 10nM T, 10nM T/6nM IGF-1, 10nM T/13nM IGF-1, 1nM E, and 10nM E, with fixed effect "antler growth stage" (30 and 60 days after the antler casting) and "length of the experiment" (2x24h and 6x24h). Second, for Experiments 3 and 4 we analyzed hormonal addition of 6nM IGF-1,13nM IGF-1, 1nM T, 10nM T, 1nM T/6nM IGF-1, 10nM T/6nM IGF-1, CA, 10nM T/CA, 10nM T/CA/6nM IGF-1, F, 10nM T/F, 10nM T/F/6nM IGF-1, 10nM E, ICI, and 10nM E/ICI, with fixed effect "antler growth stage" (15, 30 and 60 days after the antler casting) and concentration of FCS (1% and 10%).

3 Results

Experiments 1 and 2: The effect of particular hormonal treatments on the antler cell proliferation varied significantly depending on the duration of the experiment (Fig. 1). In experiment 1, compared to the control testosterone as well as estradiol significantly stimulated antler cell proliferation in both concentrations (1nM T: $P< 0.01$; 10nM T: $P< 0.001$; 1nM E: $P< 0.05$; 10nM E: $P< 0.001$). IGF-1 had no significant effect in the experiment 1 (Fig. 1). In the experiment 2 (Fig. 1), testosterone and estradiol had little or no effect (10nM T: $P< 0.05$) but IGF-1 showed

significant inhibition of antler cells as compared to the control in both concentrations (6nM IGF: P< 0.05; 13nM IGF: P< 0.001). IGF-1 also significantly inhibited the stimulating effect of testosterone (10nM T/13nM IGF: P< 0.05) (Fig. 1).

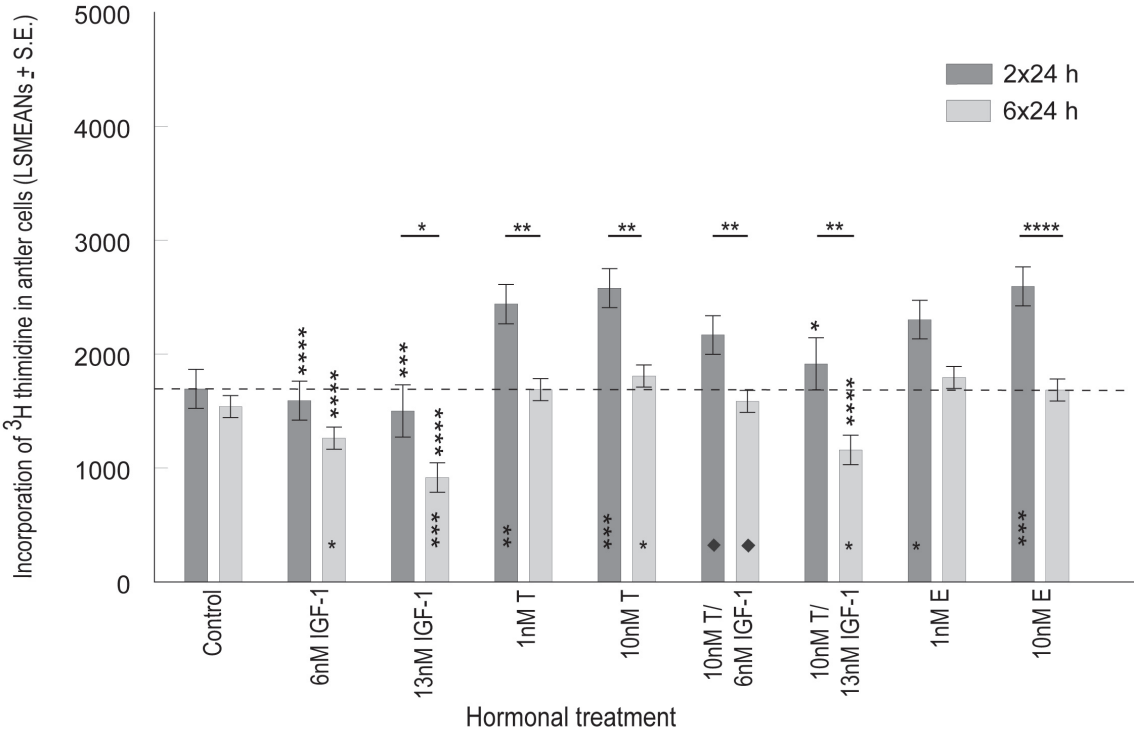

Figure 1: Experiment 1 and 2. *In vitro* proliferative response of second passage antler cells after 2x24 h or 6x24 h of hormonal treatment in 1% FCS. $\star$ $\diamond$ P< 0.05, $\star\star$ P< 0.01, $\star\star\star$ $P < 0.001$, $\star\star\star\star$ $P < 0.0001$. Stars above the lines represent differences between 2x24 h and 6x24 h of hormonal treatment. Stars inside the columns represent a difference from the control. Stars above the columns represent difference from 10nM T. Spades inside the column represent a difference from 6nM IGF-1. Only relevant significances are depicted. n≥15

Experiments 3 and 4: The effect of particular hormonal treatments on the antler cell proliferation varied significantly depending on the sampling day (Fig. 2, Fig. 3). The effect of hormones in the experiments 3 and 4 was less notable than in the first two experiments. Testosterone significantly stimulated antler cell proliferation as compared to the control on the cells from 15th day, both in experiment 3 (1nM T: P< 0.05) (Fig. 2) and in experiment 4 (10nM T: P< 0.01) (Fig. 3). Estradiol had an inhibiting effect only on the cells from the 30th day in the experiment 3 (P< 0.05) (Fig. 2). IGF-1 (concentration 13nM) showed a weak, but not significant inhibition of the antler cell proliferation on the 60th day after antler casting in both 1% and 10% FCS (Fig. 2, 3). Similarly to experiment 2, co-treatment of testosterone with IGF-1 significantly differed from testosterone alone both in experiment 3 (1nM T/6nM IGF: P< 0.05) (Fig. 2) and experiment 4 (10nM T/6nM IGF: P< 0.01) (Fig. 3). Co-treatment of CA or flutamide with testosterone antagonized

the proliferative response of the cells compared to testosterone (10nM T) alone given only in experiment 4 both on the cells from 15th (10nM T/CA: $P< 0.01$) (Fig. 3) and 30th day (10nM T/CA: $P< 0.05$; 10nM T/F: $P< 0.05$) (Fig. 3). All other significances are marked in Fig. 2 and Fig. 3. Antisteroidal treatments did not show any consistent effect on the proliferation of the antler cells neither alone nor in combinations with steroids and IGF-1.

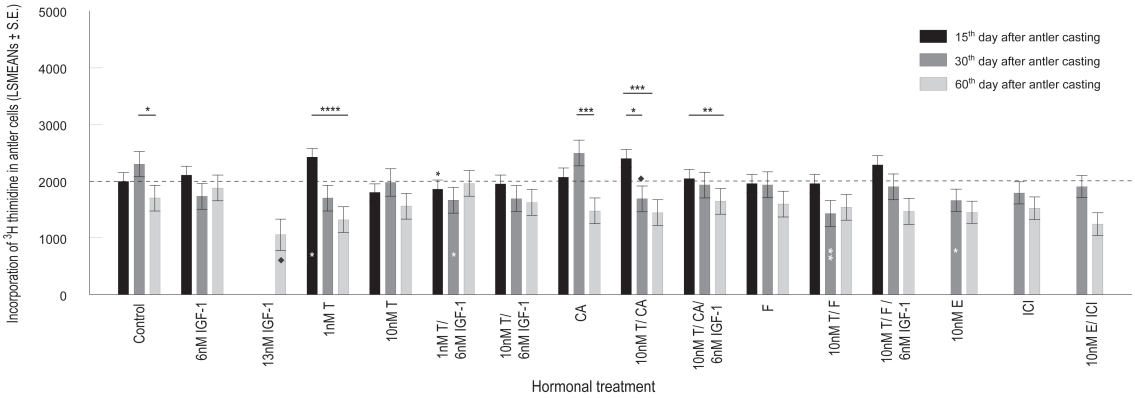

Figure 2: Experiment 3. *In vitro* proliferative response of antler cells derived on the 15th, 30th and 60th day after antler casting cultured under various hormonal treatments in 1% FCS without precultivation. $\star$ $\diamond$ $P< 0.05$, $\star\star$ $P< 0.01$, $\star\star\star$ $P < 0.001$, $\star\star\star\star$ $P < 0.0001$. Stars above the lines represent differences in the treatments among sampling days. Stars inside the columns represent a difference from the control. Stars above the columns represent a difference from 1nM T. Spades above the column represent a difference from CA and spades inside the columns represent a difference from 6nM IGF-1. Only relevant significances are depicted. $n\geq 9$

In experiments 1 and 2, the proliferation intensity was significantly dependent on the length of the experiment ($F_{(1,379)}=42.82$, $P< 0.0001$) (Fig 4A). There was no difference in the proliferation between the two sampling days. In experiments 3 and 4, the percentage of used FCS ($F_{(1,771)}=64.34$, $P< 0.0001$) (Fig. 4B) and the day of the tissue sampling ($F_{(2,771)}=102.21$, $P< 0.0001$) had a significant effect on the intensity of antler cell proliferation. In both experiments, the cells from the 15th day proliferated the most (experiment 3: compare to the 30th day $P< 0.05$ and compare to the 60th day $P< 0.0001$, Fig. 4C; experiment 4: compare to both the 30th and 60th day $P< 0.0001$, Fig. 4D).

4 Discussion

In this study, testosterone stimulated the antler cell proliferation or did not differ from the control. In some experiments, the stimulating effect of testosterone was inhibited by IGF-1. These results are in agreement with those discussed by Rolf et al. [19], but are in contrast to findings of Li et al. [21] and Sadighi et al.

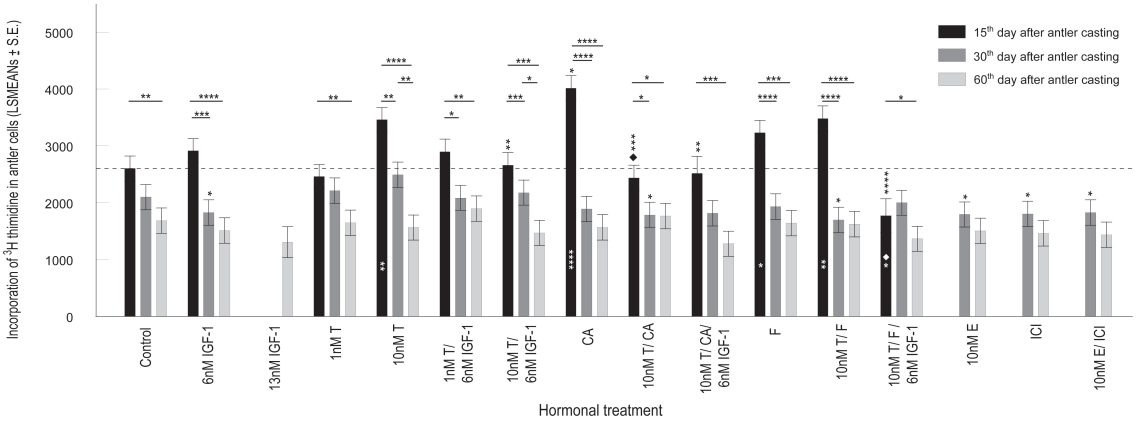

Figure 3: Experiment 4. *In vitro* proliferative response of antler cells derived on the 15th, 30th and 60th day after antler casting cultured under various hormonal treatment in 10% FCS without precultivation. $\star$ $\diamond$ P$<$ 0.05, $\star\star$ P$<$ 0.01, $\star\star\star$ $P < 0.001$, $\star\star\star\star$ $P < 0.0001$. Stars above the lines represent differences among sampling days. Stars inside the columns represent a difference from the control, stars above the columns represent the difference from 10nM T, spade above the column represent a difference from CA and spade inside the columns represent difference from 6nM IGF-1. Only relevant significances are depicted. n$\geq$9

[18] who reported that testosterone did not stimulate antler cells *in vitro* and even inhibited the mitogenic effects of IGF-1. Sadighi et al. [18] also concluded that testosterone did not sensitize antler cells to the mitogenic effect of IGF-1 *in vitro*. Although markers of differentiation were not examined neither in our, nor in their study, it was previously reported for human and rabbit bone tissue that testosterone might sensitize the cells to the differentiating effects of IGF-1 [27, 28]. That IGF-1 induces mainly a differentiation in antlers was suggested by Elliot et al. [29]. They localized the receptors for IGF-1 in the chondroblast zone of the growing antler, what implies its involvement in cartilage formation through matrixogenesis and according to Elliott et al. [29], there is no support for IGF-1 having a major role in mitosis in the antlers. Indeed, in the study of Colitti et al. [30] on antlers, chondroprogenitors did not proliferate and proliferation was barely detectable in the cartilage. On the other hand, the presence of estrogen receptors [9] and probably also the androgen receptors [12], as initiated by a presence of immunohistologically detected testosterone, were found in the fibrous perichondrium, where proliferative cells are present [30]. Moreover, Rolf et al. [31] located the antler stem/progenitor cells in the cambial layer of the perichondrium as it was also reported for other cartilagenous tissues [32]. It was suggested by Peralta et al. [33] that testosterone causes acceleration of bone growth by stimulating and subsequently depleting the source of stem cells in the cartilage growth plate and hence ceases the bone growth. However, according to Vanderschueren et al. [34] androgens alone appear insufficient to drive male periosteal bone formation. They suggested that low levels of estrogen may stimulate periosteal bone formation, but inhibit periosteal bone apposition at higher concentrations. According to their study, such dual action of estrogen on the

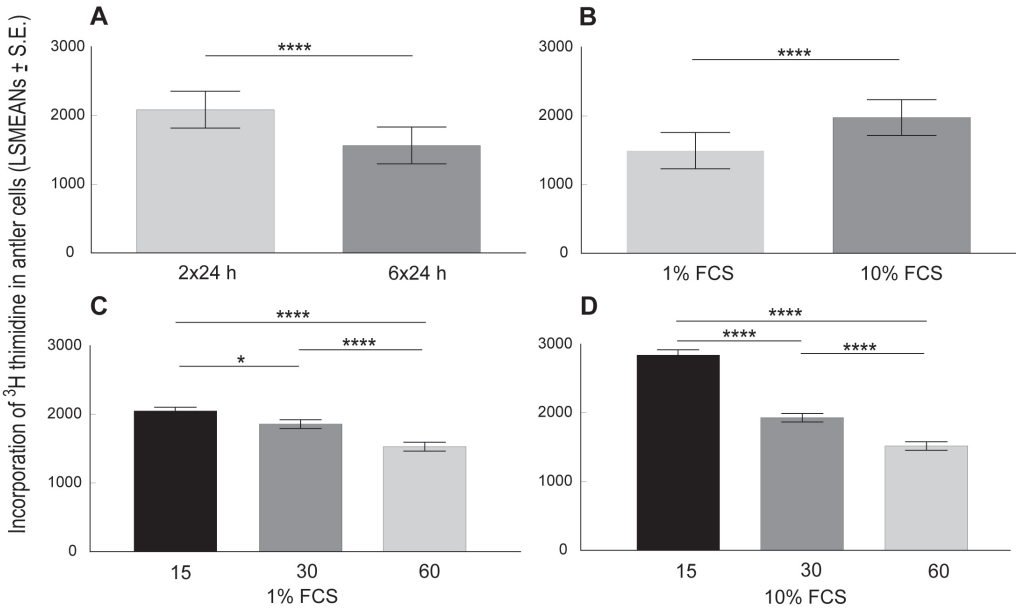

Figure 4: Factors influencing the intensity of the proliferative response of antler cells in vitro. A length of the treatments 2x24 h vs. 6x24 h – time-dependency (experiment 1 and 2). B Percentage of FCS 1% vs. 10% (experiment 3 and 4). C Differences among sampling days for cells cultivated in 1% FCS. D Differences among sampling days for cells cultivated in 10% FCS. $\star$ P$<$ 0.05, $\star\star\star\star$ $P < 0.0001$.

periosteum may be a result of the direct effect on estrogen receptors, but may also be influenced by changes in serum IGF-I.

This would support our hypothesis, that sex steroids not only cause mineralization and growth cessation at high concentrations, but also stimulate proliferation of antler cells at low systemic concentration [16, 35]. Such a dual effect of sex hormones on bone tissue has been generally accepted [33, 34, 36–41] and as Maor et al. [42] described, these effects are not only dose-dependent but also time-dependent. Time-dependency appeared also in our experiments where both testosterone and estradiol stimulated the antler cell proliferation in the short-time treatment experiment (experiment 1), but not in the long-time treatment (experiment 2).

Our results show a similar time dependency for IGF-1. The antler cell proliferation was unaffected in the short-term treatment, but inhibited in the long-term treatment what contradicts the stimulating effect of IGF-1 reported by others [17, 18, 20, 21]. This discrepancy could be explained by the fact that the effect of IGF-1 on the proliferation or differentiation is mediated by at least four different interacting intracellular signalling cascades, which are progressively inactivated as the cell differentiates and whose activity is temporarily regulated [43]. Hence, the effect of IGF-1 is highly cell-stage dependent and as reported by Price et al. [4] this might be true also for testosterone and estradiol. Other reasons for the inconsistency among results obtained from *in vitro* studies could be the influence of precultivation [25, 44] and the influence of the antler growth stage from which the samples were taken [4]. Indeed in experiments 3 and 4 we found significant differences in the

proliferation intensity among cells sampled on the 15th, 30th and 60th day. According to Patel et al. [44], the precultivation process might select a particular cell population with enhanced ability to thrive on tissue culture plastic.

To eliminate the unpredictable effect of precultivation, the experiments 3 and 4 were performed on the primary cultures. In these precultivation-free experiments, we also used selective sex steroid receptor blockers, antiandrogens CA and flutamide, and antiestrogen ICI 182,780 in order to further examine the mechanisms of sex steroid stimulations. Moreover, we extended the experiments to the sampling on 15th day after antler casting and demonstrated that the proliferative response significantly depended on the sampling day as Price et al. [4] suggested earlier.

Although we could not find a general trend in the effects of treatments compared to the previous experiments, testosterone showed a stimulating effect on antler cell proliferation on the cells from the 15th day. Generally, the cells from the 15th day proliferated with the greatest intensity and the differences among treatments were mostly pronounced when 10% FCS was used as reported by [19].

In several cases, co-treatment of testosterone with CA or flutamide led to the decrease of the proliferative response compared to testosterone alone. Kasperk et al. [26] reported the inhibitory effect of CA and flutamide on the proliferation of osteoblastic cells stimulated by testosterone *in vitro*. Indeed, after CA treatment the antler growth was completely stopped in castrated white-tailed bucks [13, 45], and stopped or was significantly reduced in castrated fallow bucks [35]. On the other hand, the inhibitory effect of CA could not be confirmed for non-castrated animals [46]. Moreover Suttie et al. [47] demonstrated that antlers of the CA-treated red deer were even larger than those of the untreated controls. However, this could be due to the dual effect of testosterone described above. Namely, the addition of CA kept the effective levels of testosterone on the cellular level low, and thus CA treatment could prolong the antler growing phase by postponing the mineralization caused by higher levels of testosterone.

In experiment 3 estradiol alone inhibited the antler cell proliferation on the 30th day, but in the rest of the experiments 3 and 4 the proliferative response was unaffected by estradiol alone or in combination with antiestrogen ICI 182,780. Estradiol has been shown to increase IGF-I mRNA levels in human bones and this effect was blocked by pure antiestrogens ICI 182,780 and ICI 164,384 [48]. Such an effect could not be confirmed by Kamanga-Sollo et al. [49] on muscle cells, who reported that ICI 182,780 enhanced IGF-I mRNA levels stimulated by estradiol, but inhibited the proliferation stimulated by estradiol. This indicates that the stimulation of IGF-1 mRNA by estradiol and its proliferative effect does not have to be interconnected. Such a mechanism could also exist in growing antler tips, where a proliferation was found only in the fibrous layer of the perichondrium, the location of estradiol receptors, but IGF-1 receptors were localized only in the cartilage layers where no proliferation was reported as described above. No proliferative effect of estradiol or ICI 182,780 in experiments 3 and 4 could therefore be explained by their effect on differentiation. However markers of differentiation were not examined and this would require further investigations. Interestingly, our preliminary experiments with ICI 164,384 alone showed a robust increase in the antler cells proliferation which was

even enhanced by adding estradiol and thus suggesting that ICI 164,384 has more agonistic, than antagonistic effect on the antler cell proliferation (unpublished observation).

The great variability observed in our study, in the experiments 3 and 4 in particular, may be also explained by the fact that the experiments where performed on mixed antler cell cultures. Therefore it is of high importance to identify the crucial cell populations responsible for the antler growth [31]. Another possible explanation is, that the usage of a high-dose of FCS in culture media might not only cause a more intense proliferative response but also emphasize some effects that are absent in a low-dose FCS or in serum-free conditions [41, 50].

The role of sex hormones in bone growth is extremely complex and all act on an endocrine, paracrine or autocrine level [1], stimulating bone growth, bone precursor proliferation and differentiation, matrix synthesis, mineralization, cessation of growth as well as maintenance of bone mass and apoptosis [1, 51]. According to our results, we do not see any reason why to restrict their function to antler cycle timing and exclude the sex hormones from the rapid antler growth phase [47]. We even could not confirm the stimulating effect of IGF-1 reported by previous *in vitro* studies [17, 18, 20].

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PAPER IV

Endocrine Relationships between Rank-Related Behavior and Antler Growth in Deer with a Focus on in Vivo Studies

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Endocrine relationships between rank-related behavior and antler growth in deer.

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Contents

1 Abstract

In this review, we analyze endocrine aspects of the relationships between antlerogenesis and rank-related behavior. The explanation of these relationships has been based on the presumption that the antler growth is regulated by hormones modulated by agonistic behavior. Originally, we assumed that these relationships are primarily testosterone dependent. In the eighties, it was reported that the insulin-like growth factor 1 (IGF-1) is the antler-stimulating hormone. This hypothesis was supposed to replace an earlier theory that the antler-stimulating hormones are either androgens or their derivatives. Here, we present historical and recent views on these issues. In particular, we analyze the arguments in favor and against the role of

testosterone and IGF-1 in antler growth and present a comparison of the results obtained across some deer species. In this context, we review and discuss experiments with castration of various deer species and analyze data from papers dealing with *in vivo* studies. We conclude that testosterone and not IGF-1 is the main antler stimulating and regulating hormone, and that concentrations of testosterone may be modified by social behavior.

2 Introduction

There are a number of reviews focused directly or indirectly on the neuroendocrine regulation of the antler cycle [1–6]. It is not our intention to present another one. The present review is based on our investigations lasting over 30 years which were focused on the relationship between dominance rank - related behavior, antler cycle timing and antler growth in deer studied in several species. With the exception of a few details, most behavioral aspects are reviewed elsewhere [7]. Here we discuss possibilities of endocrine control of the relationship between dominance rank - related behavior, antler cycle timing as well as antler growth. We concentrate predominantly on the role of supposedly "antler stimulating hormones" such as testosterone and IGF-1. Several decades ago Goss demonstrated [8] that growing antlers were more sensitive to estrogen than testosterone. As reviewed by Riggs *et al.* [9], in males and females the effects of testosterone on the skeleton are indirect, occurring after its local conversion to estrogens by aromatase [6]. Higher testosterone and lower 17 beta estradiol concentrations found in plasma compared to antler bone or antler velvet, may indicate a partial conversion of systemic androgens into estrogens in the tissues of growing antlers [10]. In summary, when we discuss testosterone, we do not extend the discussion into possible effects of other androgens [11], estrogens [6] or the possibility that estrogens may be converted locally from testosterone by aromatase [3, 4, 10, 12–14].

In a study on captive red deer *Cervus elaphus* we demonstrated that males of higher rank cast their antlers first and also tended to shed the velvet earlier than subordinate ones [15, 16]. In subsequent studies performed on the same species we found evidence that the social position and the related agonistic activity of males during the velvet period influence antler weight and length and the number of points. These studies have suggested that the antler size is a consequence of the previous social position and not vice versa [16, 17]. Later we presented evidence that in fallow deer *Dama dama* the changes in behavior, which were related to rank, modified antler growth. Males gaining a higher rank through fighting other males exhibited enhanced growth of that part of the antler that was just growing. This situation changed, if the male lost his position [18]. Detailed descriptions of the behavioral aspects of all these relationships are presented elsewhere [7].

We also attempted to explain possible endocrine mechanisms responsible for these results. Some time ago it was believed that correlations between social dominance and levels of hormones, modulated mainly by agonistic behavior exist, and

that the changes of hormone levels associated with agonistic interactions are crucial and long lasting [19]. Dominant animals were expected to have generally lower pituitary/adrenocortical activities than submissive animals living with them. Males with a dominant position usually tended to have elevated androgen levels [20]. Conversely subordinate status seemed to be associated with lower androgen secretion and increased levels of glucocorticoids [21, 22]. Therefore, since the very beginning of our investigations, we assumed that the mechanism of the relationship between rank position and antler cycle timing lies in presumably elevated levels of testosterone in dominant males and decreased concentrations in subordinate individuals [15, 16].

Testosterone was a candidate hormone for several reasons. First, we believed testosterone was involved in the regulation and development of antlers [3, 15, 16, 23]. Secondly, testosterone promotes the development of secondary sexual characteristics across many species of the animal kingdom [24, 25]. Thirdly, as mentioned above, testosterone concentrations can be modified throughout behavior due to a feedback. However, the first prediction, that testosterone is involved in the regulation and development of antlers, was not that clear.

3 Antler Development

3.1 Antler Growth and Testosterone

Antler development is a dynamic multi-factorial process reflecting changes in the environment. No wonder, generations of deer biologist have attempted to answer the question of what is the main antler growth hormone [3]. As shown in many studies, antler development is invariably associated with an increase of testosterone concentrations across cervid species, such as white-tailed deer, *Odocoileus virginianus* [26–29], Columbian black-tailed deer, *Odocoileus hemionus columbianus* [30], roe deer, *Capreolus capreolus* [31–33], red deer [34, 35], axis deer, *Axis axis* [36, 37], fallow deer [38–42], rusa deer, *Cervus (Rusa) timorensis* [43], Eld's deer, *Cervus eldi thamin* [44], and pudu, *Pudu puda* [45]. All these reports provide good arguments in favor of accepting testosterone as the hormone supporting antler growth. On the other hand, it has been also accepted that increasing seasonal levels of testosterone cause cessation of antler growth by mineralization of the antlers, shedding of the velvet, and the attachment of the dead antler to the pedicle, as observed across various deer species [3, 46, 47]. However, a considerable increase in testosterone concentration which causes the mineralization of antlers and the shedding of velvet occurs only after the cessation of antler growth and the completion of antler bone development [3, 48]. The antler casting is generally associated with a rapid decrease of seasonal levels of testosterone [3, 8, 46]. Nevertheless, already in the classical experiments with castrated white-tailed deer, Aub and Wislocki [46, 49] stressed the importance of testosterone in antler growth induction. By giving testosterone to their males, which had never had antlers as a result of castration as fawns, they induced antler growth. Similarly, administration of testosterone to ovariectomized

female deer, or a blockade of ovarial function of a doe with an antiestrogen, caused them to grow antlers [46, 49, 50]. In roe deer, low levels of testosterone initiated not only growth of the pedicles but also a subsequent growth of antlers [51]. On the other hand, high testosterone levels prevented any growth of pedicles on the same deer. Later on [52] a small amount of androgens given to male sika deer in food during the velvet period stimulated their antler growth. In the velvet antlers of white-tailed deer, Bubenik *et al.* [53] localized immunohistologically testosterone in the prochondral blastema layer, i.e. in the preosseous cartilaginous zone responsible for cartilage matrix synthesis but not in the ossification zone. The authors suggested that the potential importance of this hormone is in the bone matrix synthesis and not in ossification. At that time it was already accepted that in humans a low concentration of testosterone can stimulate bone growth, whereas larger doses can be inhibitory [54]. That inspired Brown *et al.* [55] to perform a study in white-tailed deer. They determined the relationship between serum androgen concentrations and changes of relative bony density in the antlers and long bones of male deer sampled twice a week during the antler growth period. Circulating androgen concentrations increased over the entire antler-growth period, as did the relative bone mass (RBM) coefficients of the antler. Brown and co-workers found positive correlations between increasing androgen concentrations and increasing antler RBM and negative correlations between androgens and decreasing RBM of the metacarpus. The antler RBM coefficients continued to increase after polishing of antlers, but metacarpus RBM did not change after velvet shedding. Two castrated deer were injected subcutaneously with 1g of testosterone and sampled every other day. Similar but smaller changes occurred in RBM values of the metacarpus and developing antler in castrated deer injected with testosterone. Their experiments on white-tailed deer were in agreement with earlier results published in roe deer by Tachezy [51]. In both species the authors indicated that low serum concentrations of testosterone can stimulate bone growth, while higher levels will cause inhibition [51, 55].

Based on these data it was hypothesized that new antler growth may be initiated by a short reactivation of reproduction and hence resulting in a testosterone pulse (Fig. 1) [3, 15, 56, 57]. This suggestion was later supported by studies showing a short-termed peak of testosterone in the period of antler regrowth in several deer species such as roe deer [32, 33, 58], white-tailed deer [27, 59, 60], wapiti [61], red deer [62–64], fallow deer [38, 41], black-tailed deer [30], etc.

In summary, we interpreted a link between rank - related behavior and antler casting as follows: We would expect that the effect of testosterone on the initiation of antler regrowth and consequent antler casting is modulated by behavior and thus is being involved in the formation of actual hormone levels. The short reactivation of reproduction and hence testosterone pulse (Figure 1), which probably triggers antler regrowth [3, 15, 57], occurs during the period of lowest seasonal levels of testosterone. The more dominant males have earlier, higher and more frequent testosterone pulses during low seasonal concentrations of testosterone. This testosterone range corresponds to the Tachezy' s [51] and Brown *et al.*'s [55] 'low amount' of androgens. A new antler bone growth may thus be initiated more vigorously and the antler casting in species like red deer (Group A type casting in [16]) may occur

earlier [15, 16]. Also in deer males such as seen in white-tailed deer (Group B type of casting in [16]), new antler growth of dominants may start earlier even though antler casting had occurred later. More about the different group types of casting is presented elsewhere [16].

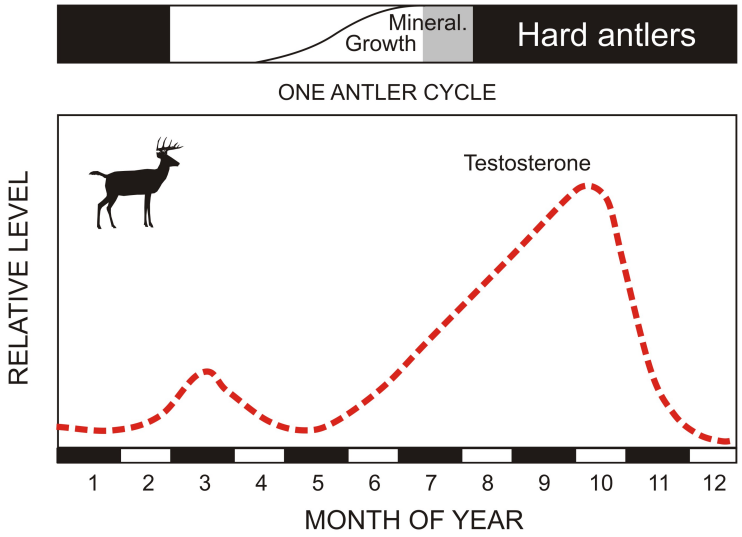

Figure 1: Stylized time course of hormonal levels during the antler cycle of a white-tailed deer (*Odocoileus virginianus*). Adapted with permission from Bubenik [3].

To explain the relationship between rank - related behavior and antler cleaning seems to be less complicated because antler polishing occurs in the time of seasonally elevated testosterone concentrations. The stimulatory effects of social interactions among dominant males probably elevate the levels of testosterone, while the interactions elevate glucocorticoids and depress testosterone levels in subordinates [16]. As a result, antler cleaning may occur earlier in dominants and later in subordinates. Many authors have suggested that antler cleaning dates are fully dependent on age, such as it happened in red deer [65–67] and in other cervids [68]. With the exception of fallow deer [69], spike-antlered deer polished antlers later than fork antlered males in red deer [65, 67, 70, 71], white-tailed deer [68, 72, 73], and moose *Alces alces* [74]. However, both the earliest and the latest cleaning dates were also observed among yearlings in fallow deer [75, 76], in white-tailed deer [73], moose [74], and in our red deer herd [77]. In contrast to some of the above mentioned reports, the alpha males were not usually the oldest ones in our study population [78]. And, indeed, the antler casting/cleaning times of individual males appeared to be dependent primarily on their social status and the influence of age was of secondary importance [15]. Hence we concluded that for the above alternating results this variation is due to different opportunities for social grouping. It brings a differential social stimulation of the process, as well as diverse opportunity to be stressed [16].

In addition, the relationship between rank - related behavior and antler growth seemed logical. The physiological consequence of the male's behavior on his antler growth may have acted since the beginning of the velvet period. The more dominant a male is, the higher the seasonally attained levels of androgens within the actual physiological range and the greater the enhancement of antler formation [16].

3.2 Antler Growth and IGF-1

In the mid-1980s Suttie *et al.* [34] compared the seasonal variations of hormones with the progress of antler growth in red deer (Figure 2). Based on their data, they concluded that the insulin-like growth factor 1 (IGF-1) is the antler-stimulating hormone. In the subsequent study they aimed to answer the question whether IGF-1 acts on antler growth through the general blood circulation or if it is of local origin. They completely cut growing antlers and observed a significant elevation of plasma levels of IGF-1 in the non-antlered stags compared with normal antlered stags during the antler growth period. Therefore they concluded that the growing antler is a target organ for IGF-1 and that the prevention of antler growth removed a population of IGF-1 receptors [79]. A similar increase of IGF-1 concentrations corresponding to the progress of antler growth was also reported by Schams *et al.* [80] in roe deer, and Reyes *et al.* [45] in pudu.

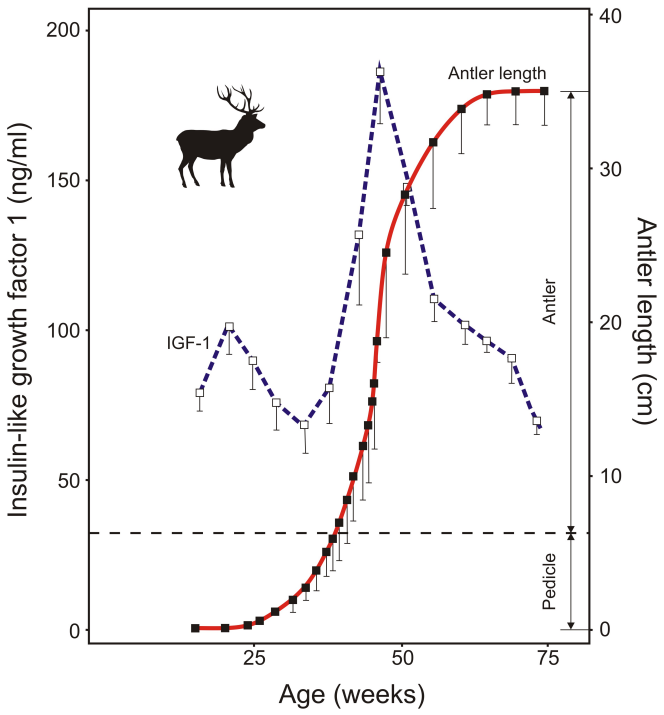

Figure 2: The relationships between insulin-like growth factor 1, antler length and calendar time in red deer (*Cervus elaphus*) yearlings. Adapted with permission from Suttie et al. [34].

The hypothesis that IGF-1 is an antler-stimulating hormone displaced an earlier notion which suggested that the antler-stimulating hormones are androgens, particularly testosterone or its derivatives [3, 4, 15, 53, 55]. In 1992 Suttie *et al.* [81] stated that "The male can be considered almost a functional castrate for the first few weeks of velvet antler growth".

3.3 Antler Development and Castration

The principles of the effect of castration were first described by Aristotle [8, 57] and confirmed in many modern experiments performed since the 1930s and 1940s, either in Europe [82] or North America [46]. More recently, several laboratories focused on the evidence indicating that testosterone does not play any significant role in the stimulation of antler growth. They neutered male deer either by administration of androgen receptor blocker or by surgical castration.

To elucidate the participation of testosterone in the formation and maturation of growing antlers, the influence of antiandrogen cyproterone acetate (CA) on the antlers was studied in several species. In a pioneering study on white-tailed deer yearlings [23], the growth period of antlers, after CA application, was delayed by three months, in comparison to intact controls. The CA treatment, introduced in the second half of the normal antler growth, resulted in an incomplete mineralization of the antler beam, delayed velvet shedding and a diagonal sequestration of the top portion of the antlers. We observed an incomplete formation and mineralization of the Haversian systems. The antler structure of the tips was similar to that observed in white-tailed castrates [23]. Schams *et al.* [83] then reported that CA treatment did not inhibit antler growth in intact roe bucks. It only delayed the velvet shedding until after the end of the treatment period. When CA was applied outside of the season of antler casting, CA treatment did not prevent antler growth nor the attainment of a species-specific antler shape in intact fallow bucks [84, 85] and red deer stags [86]. In all these studies antler growth was nearly normal and of species-specific shape. Similarly, the morphology and histological structure of antlers grown after surgical castration of adult fallow deer bucks also remained comparable to intact individuals. Only the velvet was not shed [87]. The general believe of various researchers was that antler morphogenesis proved to be non-androgen dependent [84]. On the other hand, non-species specific shape of antlers were described in fallow deer castrates [42], white-tailed castrates [88], white-tailed deer after CA [23], in hypogonadic white-tailed deer [89, 90], and presumably hypogonadic California mule deer *Odocoileus hemionus californicus* [91], etc. Also the formation of Haversian systems in the growing antlers was substantially affected by CA [92]. Despite these findings some authors still insisted that species-specific antler growth can occur without testosterone stimulation [84, 86, 87]. More recently, Kierdorf *et al.* [93] discussed in detail that the antlers of fallow deer castrates show histological signs of immaturity due to the lack of androgens. The overall antler growth, attainment or not of a species-specific shape of the antlers, and the question of the maturation of antler bone, is rather complicated process. The discussion how individual stages of these processes are influenced by androgens is beyond the topic of this review.

3.4 Antler Development and IGF-1 in Different Deer Species

The concept that velvet antler growth can occur without testosterone stimulation during the period of velvet growth challenged our speculation about the hormonal base of the relationships between rank - related behavior and the antler development [3, 4, 15, 16]. Therefore, we focused first of all on finding the possibility of a link between rank - related behavior and IGF-1. In a study on pudu [94], the analysis revealed that from September to November (the second part of the antler growing period and the time of establishing territories) the IGF-1 levels of dominant males were significantly higher than those of subordinate males. This finding supported the concept that IGF-1 is the antler stimulation hormone. Concurrently, however, these results induced two kinds of doubts. The first doubt was associated with very little evidence about the possible relationship between IGF-1 levels and dominance. At the same time when we performed our study on pudu, Sapolsky & Spencer [95] reported that IGF-1 was suppressed in their socially subordinate baboons. Though, as far as we know, since that time no other study which would show lower concentrations of IGF-1 in subordinate individuals has been published. The second doubt was even more serious.

As mentioned earlier, we determined an increase of IGF-1 concentrations corresponding to the progress of antler growth in pudu [45]. When we looked at the increase of IGF-1 from the point of view of the male dominance, the increase of IGF-1 corresponded with the progress of antler growth in dominant but not subordinate males [94]. Still all males produced antlers (Figure 3). More than that, analyzing hormonal profiles in reindeer males and females, we found that a trend of elevating IGF-1 was associated with growing antlers in males but not females (Figure 4), either pregnant or non-pregnant [96].

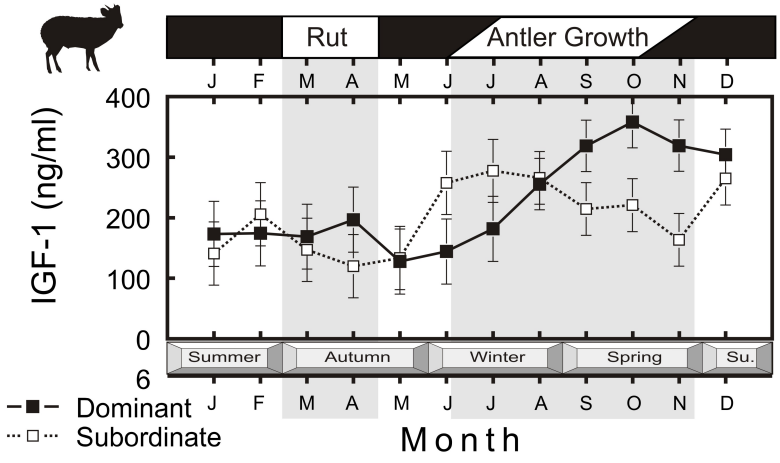

Figure 3: Adjusted means (± S.E.) of seasonal levels of IGF-1 and testosterone according to rank of pudu (*Pudu puda*) males. Shaded areas represent the time of the rut and the velvet antler period. Adapted with permission from Bartoš *et al.* [94].

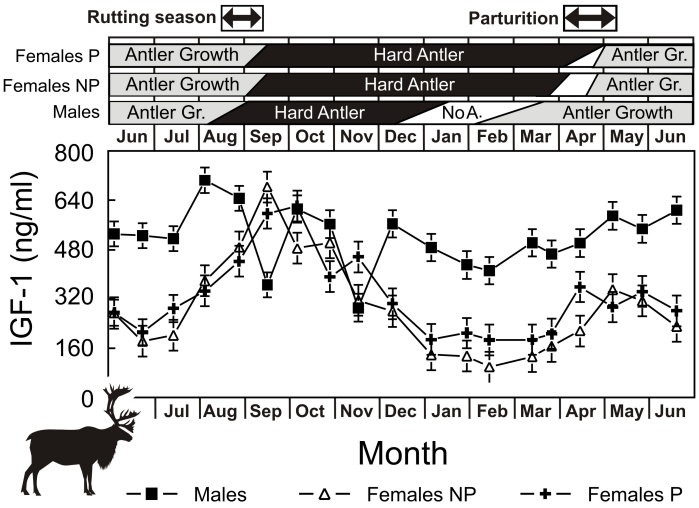

Figure 4: Adjusted means (± S.E.) of seasonal levels of IGF-1 in male and pregnant and non-pregnant female reindeer (*Rangifer tarandus*). Adapted with permission from Bubenik *et al.* [96].

4 In Vitro Cultivation of Antler Tissues in Relation to IGF-1 and Testosterone

The role of IGF-1 and androgens, particularly testosterone, in the proliferation of antlerogenic cells *in vitro* is still not satisfactorily elucidated and remains controversial. In antler cell culture experiments, IGF-1 was found to stimulate the proliferation of antlerogenic cells from the antler tip [97, 98] and played a role in the regulation of antler growth [6]. Similarly, Li *et al* [99] showed that IGF-1 stimulated the proliferation of antlerogenic cells from the antler pedicle in various ossification stages. On the other hand, in our recent study IGF-1 did not have any effect or even inhibited proliferation of cells obtained from the antler tip [100].

Reports on testosterone also vary greatly. Sadighi *et al.* [101] did not observe any stimulating effect of testosterone on antler cells proliferation. Moreover they showed that testosterone at certain levels suppressed the mitogenic effects of IGF-1 on the antler-tip cells. Identically, in the experiments of Li *et al.* [99] testosterone alone did not show any mitogenic effects, but in the presence of IGF-1it increased proliferation at certain ossification stages of the pedicle. On the other hand, Price *et al.* [2] stated that testosterone will induce the proliferation of cells cultured from antlers depending on the stage of antler growth and the stage of cell differentiation. Similarly, in the study of Rolf *et al.* [102] sex hormones (testosterone, dihydrotestosterone) stimulated the proliferations even in high concentrations. Finally, our experiments also showed proliferative effect of testosterone [100].

Some of the above-mentioned studies were performed in the serum-free conditions

[103], which could be regarded as physiologically optimal, while others used serum in various concentrations [97], because it is difficult to keep the tissue growing without it. Both approaches have supporters as well as critics. Clearly, this could be one of the major reasons of the discrepancy between the studies. Even the presence of serum during the precultivation and passaging [103] could be important as this could influence later proliferation response of cell cultures [104].

In conclusion, the antler cell culture experiments have been performed under widely variable conditions. Cells were obtained from pedicle [98, 105] or antler [101–103, 106], from different stages of antler development and growth, cultivated either as primary cultures [106] or after two passages [101–103, 105], grown in medium containing fetal calf serum [98, 101–103, 105, 106] or partially cultured in serum free conditions [98, 101, 103, 105].

As stressed by Borenstein *et al.* [107] obvious limitation of a "narrative review", such as that in the previous paragraph, is the subjectivity inherent in this approach. Different authors might use different criteria for deciding which studies have relevance and which do not. To overcome this, we applied meta-analysis using Comprehensive Meta-Analysis software (Biostat, Englewood, NJ). Our goal was to analyse all studies refering to the effect of either testosterone or IGF-1 on proliferation of antler cells *in vitro* in order to see if there is a common robust trend of the effect. The studies focussed on an interaction between the two hormones were excluded. For testosterone we collected five studies. Each study was comprised of several experiments which entered the analysis as subgroups. There were 16 subgroups in [100], 4 in [98], 5 in [105], 26 in [102], and 8 in [101]. For comparing the studies investigating the effect of IGF-1, 7 studies were available with the number of subgroups 12 in [100], 32 in [98], 3 in [105], 15 in [97], 3 in [1], 20 in [103], and 6 in [101]. In the analysis, the subgroups were always combined within the study. All the studies were based on comparing the means with standard deviations and sample size between treated and control groups. Assuming that the true effect size varies from study to study, and the summary effect is our estimate of the mean of the distribution of effect sizes, we used the random-effects model [107]. We applied the Q statistic test, Tau-squared, Tau, and inconsistency index (I^2) to estimate the heterogeneity [108] of individual studies contributing to the pooled estimate [107]. The results of the meta-analysis are shown in Figure 5 (top for testosterone and bottom for IGF-1). Studies investigating the effect of testosterone reported much more variable and contradicting results either within or between studies than those of IGF-1. The overall summary showed a significant trend for IGF-1 (favouring the IGF-1 treatment in comparison to control) and not for testosterone studies. As such it would somewhat contradict the *in vivo* studies. On the other side, all measures of heterogeneity are very high in both meta-analyses with I^2 suggesting inconsistency across the findings of the studies reaching over 80% of total variation across studies due to heterogeneity for testosterone and nearly 100% of that for IGF-1. This calls the overall summary results in question and further analysis is therefore required [107]. The main difficulty here is that "antlerogenic cells" refers to an ill-defined, mixed population of cells taken from the tip of growing antlers. It is probably a mixture of mesenchymal cells, chondroprogenitors, chondrocytes and possibly even

osteoprogenitors and osteoblasts. There is no reason to assume all these cells show the same reaction to IGF-1 and testosterone. Moreover, we have recently reported that for example the individuality of the animal from which the antler tissue was taken, and also various other factors, significantly affected antler cell proliferation [104]. However, some of these factors, if not all, were omitted in most of the earlier studies. This may be another reason why no obvious general trend across the studies has been as yet discovered. An alternative possible explanation is that testosterone plays significant role in antler growth, but through an indirect way (acting on another molecule or aromatized to estrogens, etc.) *in vivo*. These systems are not available in an *in vitro* condition and that is why an effect of testosterone could not be expressed so much.

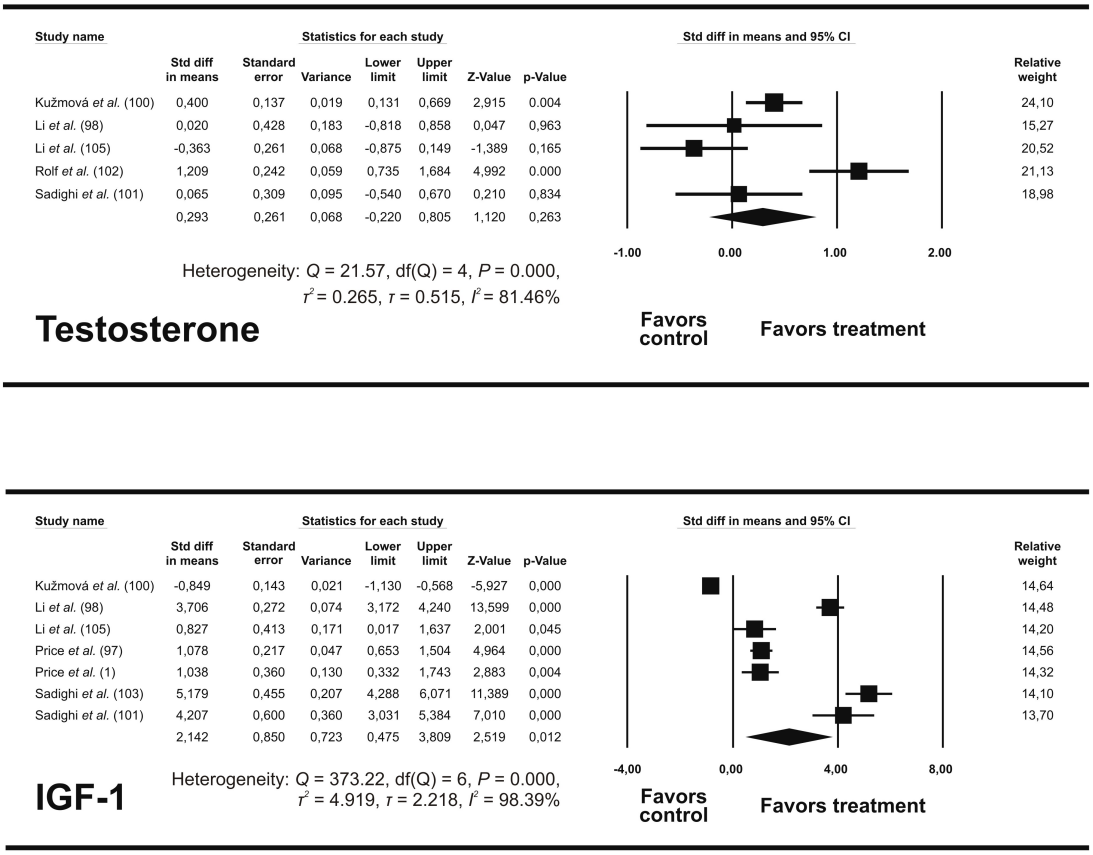

Figure 5: Forest plots showing meta-analysis of studies reporting possible effect of testosterone (top) and IGF-1 (bottom) on antler tissue proliferation *in vitro* expressed as the means with standard deviations and sample size between treated and control groups. Mean differences are shown with 95 per cent confidence intervals. The size of the marker indicates the weight of the study. The summary effect is displayed by the diamond.

In summary, dispite the fact that the *in vitro* studies have potential to exclude

a number of confounding factors that operate *in vivo* and despite the tremendous development of this field, the results of such studies which focused on hormonal regulation of antler growth should be taken with extreme caution [104]. In general all important factors affecting antler cell proliferation *in vitro* should be first satisfactorily investigated and the methodology generally standardized. Furthermore, we have not found any link between behavior and IGF-1 concentrations in any of our previous studies except one [94], although we did investigate it. Hence, combination of all factors presented in this section led us to decide not to use the results obtained from antler cell cultivation in the further discussion.

5 Antler Development, IGF-1, Testosterone and "Double Castration"

In the above-mentioned studies working with deer castrates [84, 86, 87], the detection limit of testosterone was about 0.1 ng/ml plasma. Therefore the question of a possible biological function of testosterone at concentrations below or near 0.1 ng/ml has not been addressed. We got our inspiration from Rivest *et al.* [109]. They have demonstrated that alterations of various biological processes are triggered by the fluctuation of the day length regardless of the light intensity. If there was any variation in testosterone below the level of 0.1 ng/ml, we postulated that analogically, those androgens still might have been of biological relevance as the sensitivity of androgen receptors is enhanced during the period of low circulating levels. It is also a well known fact that in several biological systems, hormones are effective not only in quantities of ng/ml but also in pg/ml, such is the case of pineal hormone melatonin day-time levels of which are ranging from 10 to 20 pg/ml [110]. In order to test this hypothesis, we designed an experiment with the code name "double castration". The aim of that study was to test experimentally the effect of a complete or an almost complete withdrawal of any androgen action on antler growth in fallow deer by comparing surgically castrated fallow bucks with surgical castrates treated with high doses of the CA. High doses of CA were given in order to block the action of androgens produced in the adrenal cortex. We tested the following two hypotheses: (i) If new antler growth is induced by a short-term pulse of testosterone, then such a pulse should be detected in animals producing antlers even below the level of 0.1 ng/ml; (ii) If androgens were required for antler growth, then the CA-treated animals should not produce antlers at all or only a reduced antler growth should be observed, as compared to surgical castrates [41].

We divided twelve yearling fallow deer bucks into two groups of six animals each. The experimental animals (CA group) were injected with high doses of CA while the control bucks (Control) were given a vehicle solution (castor oil) only. Treatments were performed two days before castration, at the day of castration (day 0) and afterwards at two day intervals until day 22, when all of the animals had cast their antlers. Blood samples for hormone analyses were taken at the same time as the treatment and the antlers, if any were produced, were measured. Thereafter CA

treatment and blood sampling were continued at weekly intervals. Testosterone was measured by EIA [111] reaching a sensitivity level between 1 and 10 pg/ml plasma.

After surgery, all animals cast their antlers 12 to 22 days post castration. New velvet antler growth was first observed more than one month after casting, around the time when new antlers usually start to develop in intact animals. Antler regrowth occurred in all controls and the antlers produced by these animals were much larger than those of CA-treated castrates. Only four of the six CA-treated castrates initiated antler growth at the same time as the controls, while in the remaining two no regrowth was observed until day 196. At that time, a unilateral (left side) antler formation started in one of these bucks, whereas in the other no antler growth at all occurred until the end of the experiment. Thus we demonstrated that also in fallow deer CA application to surgical castrates has a potential to prevent any antler growth as shown earlier by Bubenik [3] in white tailed deer. The antlers produced by our Controls were much larger than those of CA-treated castrates (Figure 6).

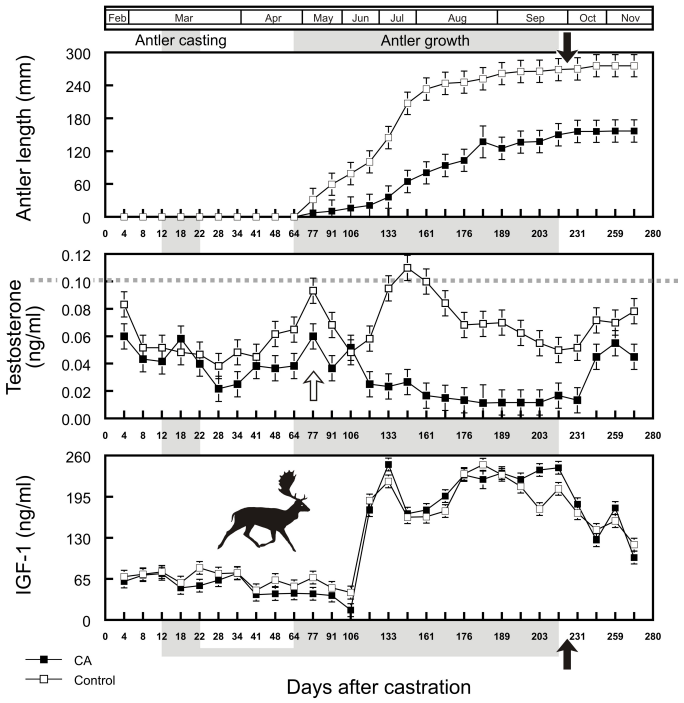

Figure 6: Antler length, testosterone and IGF-1 concentrations (adjusted means ± S.E.) between 4 and 273 days after castration of fallow deer (*Dama dama*) yearlings in Cyproterone acetate (CA) and control groups. (The white arrow indicates a short-termed elevation of testosterone associated with the initiation of antler regrowth; black arrows indicate the time of the last treatment with CA and with castor oil; the dashed horizontal line in the middle frame indicates the level of 0.1 ng/ml.) Adapted with permission from Bartoš *et al.* [41].

Moreover, we have addressed the question whether the onset of antler regrowth is triggered by a short-term pulse of androgens. In both groups we found a significant temporary increase in testosterone levels around the time of the onset of

antler regrowth, the elevation being more pronounced in the control bucks (Figure 6, middle, white arrow) [41]. It can be argued, however, that this increase should have occurred at the sampling date preceding the one at which it was found. On the other hand, in this situation one week interval for sampling could have been too long. Thus, our elevated testosterone levels could be in fact already decreasing from the previous peak not detected due to the time interval. As shown in Figure 6, the short-termed testosterone increase putatively initiating antler regrowth may be relative. If the scaling of the graph for the seasonally lowest values is the same as that for maximal seasonal concentrations, the relative increase, if present, would be hardly recognized. This is perhaps, why in fallow deer bucks Asher *et al.* [112] and in red deer yearlings Suttie *et al.* [81] did not find any variation in a testosterone surge during the antler growth initiation. In both studies the sampling was performed so often that the peak should not be missed if it ever existed. The blood samples were withdrawn at intervals of 20 [112] or 30 min for 24 h [81] from each male. However, a temporary increase of testosterone during the initiation of antler growth was indicated in the former study [112]. The levels detected below 1 ng/ml suggest that such an increase in the testosterone pattern of the intact bucks G3 and G32, seen in Asher *et al.*'s Figure 2 [112] is not well visible when the highest levels in the same graph reached nearly 6 ng/ml. Because the study was aimed a different way, no wonder this short-termed increase was neither mentioned nor analyzed. Figure 7 is based on the same data originating from our study as Figure 6 [41], with only a two day backward extension before the animals were castrated (not shown in the paper). A variation in testosterone levels is not seen here. This is because testosterone concentrations before and at the time of castration are 10 times higher than after castration. When pooled, the pre-castration data strongly shape the calculation of standard errors during the post-castration period. These make the standard errors much greater than the average values. As the result, when analyzed statistically, no variation in the post-castration period could be significant. In the otherwise existing differences in the post-castration period, the means of the two groups would thus be completely masked. In the "double castration" experiment we also tested if the growth process itself requires low levels of androgens. Antlers produced in that study were much larger and androgen levels were significantly higher in controls than in the CA bucks (Figure 6). Hence, we concluded that in fallow deer a minimum threshold level of androgens, testosterone in particular, is a necessary prerequisite for antler growth to occur. Moreover, within the low range of plasma testosterone concentrations recorded in our experimental animals, we were able to demonstrate an increase in an antler growth rate with increasing testosterone levels (expressed as areas under the curve), i.e. a dose related response of the antlers to testosterone. An increase of antler length between successive sampling sessions correlated with the change over the same period in testosterone ($r_s = 0.64,\ P < 0.02$) but not IGF-1 ($r_s = 0.13$, NS) [41].

It is obvious that our experimental design constituted an extremely artificial situation for the bucks. Nevertheless, change in testosterone concentrations was positively associated with the rate of antler growth in castrated fallow deer without CA treatment [42] but also with the antler growth in intact red deer [35]. (The

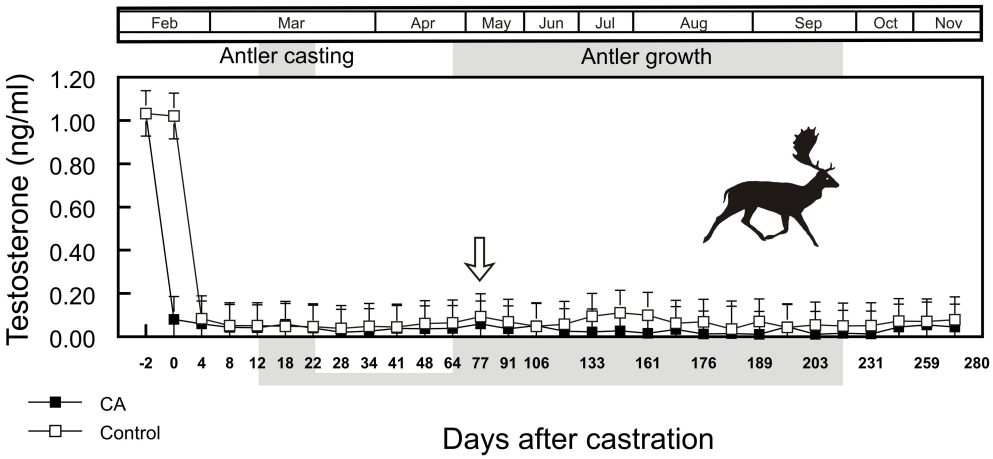

Figure 7: Testosterone concentrations (adjusted means ± S.E.) between -2 and 273 days after castration of fallow deer (*Dama dama*) yearlings in Cyproterone acetate (CA)-treated and control groups. (The white arrow indicates the time after castration. The short-termed elevation of testosterone is associated with the initiation of antler regrowth, as shown in Figure 6.

change in concentration rather than the actual testosterone concentration was used in the analyses because it reflects better the dynamic of the process and helps to avoid overlooking time shifts if there were any.)

6 Discussion

Because we wanted to keep the time context of this review, we reviewed mostly literature of the period in which the discussed studies were published. Hence, it is a rightful claim to ask how our conclusions agree with the recent views and scientific evidence. In general, our conclusions are in full accord with recent reviews on physiology of bone formation in humans and/or laboratory rodents [113, 114]. It has been repeatedly shown that the direct effects of androgen on the skeleton are complex and both stimulation and inhibition of bone formation were observed *in vivo* [115, 116]. Androgens increase bone mass in specific skeletal compartments through effects on bone cells, enhancing the activity of bone-forming cells, the osteoblasts, but inhibiting that of bone resorbing cells, the osteoclasts [117]. Androgens can stimulate the skeleton not only through direct activation of the androgen receptor, but also indirectly, after aromatization to estrogens and subsequent activation of estrogen receptors [12–14]. As Callevaert *et al.* [13] reviewed, in peripheral tissues including bone, testosterone can be irreversibly converted to the more potent dihydrotestosterone. In addition, testosterone can be converted to 17 beta estradiol and subsequently activate estrogen receptors. Therefore, androgens might activate both, the androgen and the estrogen receptors, depending on the relative activities of the responsible enzymes. These enzymes are all expressed in bone tissues, thus

suggesting that the local hormone synthesis might be important [13].

Similarly to our experiments on castrated fallow or intact red deer [35, 41], studies with male mice also concluded that androgens [118] and estrogens [119] enhance skeletal growth independently of either systemic or local IGF-I production.

IGF-1 is recognized as an important regulator of bone formation. Various *in vitro* studies reported that IGF-1 regulates proliferation and differentiation of bone cells [120–122]. *In vivo*, IGF-1 was shown to regulate growth and density of bones [123, 124]. However as demonstrated by Ciarmatori *et al.* [125] the effect of IGF-1 on proliferation and differentiation of chondrocytes on the cellular level is mediated by at least four signaling pathways which are progressively activated and inactivated as chondrocytes differentiate. This clearly shows that the anabolic effect of IGF-1 is complex and cell stage-specific. Besides, the systemic effect of IGF-1 is regulated by its binding proteins which have both a stimulating and inhibiting effect on osteoblast function [126]. Since antlers are bones [46, 51, 127], we may ask again the question why should the regulation of antlers evolve differently from that of other bones? Therefore, in the view of the presented data, we concluded that it is basically testosterone (possibly in an interaction with other steroids) and not IGF-1 which is primarily responsible for the intensity of antler growth in deer males [35].

At the same time it must be stressed that we are not maintaining that testosterone is the only hormone solely regulating antler growth. There is no doubt IGF-1 is an important hormone involved in regulation of body growth [128, 129]. Clearly, it is also somehow involved in regulating antler growth, directly or indirectly, either as such or in an interaction with testosterone [35, 130], as may be other steroid and peptide hormones involved in bone growth and modeling [2, 6] which should be further investigated.

Perhaps, this review may be found biased in favor of the literature dealing with the role of testosterone in antlerogenesis. Part of the bias is based on an imbalance of the previous debate in the literature. On one hand, studies involved in the debate after the pioneering article of Suttie *et al.* [34], still endorsing or at least admitting that testosterone is an antler stimulating hormone, usually also studied IGF-1 effect on antler tissues *in vivo* [35, 41, 80, 94, 96]. On the other hand, the introduction of IGF-1 as a possible antler stimulating hormone has not been confronted with the minimal variations of testosterone levels nor with an earlier arguments favoring testosterone [34, 79].

Nevertheless, once we accept that testosterone is primarily responsible for the intensity of antler growth, we can explain the endocrine aspect of the relationship between rank - related behavior and antler growth in deer. In a recent study on red deer we discovered that small changes in social conditions can profoundly affect the relationship between their rank and testosterone levels. Adding much younger and weaker sparring partners into the experimental group of adult males altered the agonistic behavior of the adults. Adult males targeted preferentially their attacks on individuals much lower in the hierarchy. Experimental male deer with a higher social rank had lower levels of testosterone when they were in a group of adults. After an addition of young conspecifics, it was just the opposite. Stress from competition with equally strong group members was reflected in cortisol concentrations. In a

situation when adults were alone, they had elevated cortisol concentrations. These concentrations declined after the youngsters were added. Thus, changing the social environment of adult red deer males resulted in a change of the relationship between rank and testosterone and also cortisol concentrations despite the fact that the rank position of the adults itself did not change [131]. This was reflected in an alteration of their antler development (unpublished).

7 Perspective

A further investigation thus should focus on understanding the social relationships among male deer during the period of antler growth. Especially, we should concentrate on facts how to record most objectively the social structure of a male deer group. This should be done in order to foster further analyses linking the antler development to concentrations of hormones. It will also bring a better understanding of the role of antlers in mate selection [7].

8 Acknowledgement

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