# ANTLER GROWTH IN DEER

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## General introduction

Since the time of Aristotle, at least, people have been interested in antler growth and regeneration and its underlying causes. Much of what we now know about antler growth has come from histology, organ ablation/hormone replacement studies typical of classical endocrinology, and hormone analysis in serial blood samples, with or without a prior challenge from a releasing hormone. However, in the last few years novel approaches to the study of antler growth have begun. This manuscript describes three of these, namely A) i) antler tissue incubation with hormones, ii) *in vitro* culture of antler cells, and B) serial radiography of antlers in deer whose plasma hormone profiles were also measured.

## PART A Growth factors and antler tissue growth

### Review

It is important to distinguish between hormones/factors which influence antler size and those which influence whether or not the antler will grow or regenerate. A great deal of research points to the fact that the annual cycle of antler growth and replacement is controlled by testosterone. Plasma levels of testosterone are very low during velvet antler growth, rise at the time of the cleaning of the antler of velvet, are high during the breeding season and fall to essentially zero levels at casting (Lincoln 1971). Studies of castrate deer have shown that if they are in hard antler at the time of castration, the antlers are cast and if they are in velvet antler they remain so. Antlers regrow after castration induced casting, in velvet, but are never cleaned unless a gonadal steroid is administered. If this gonadal steroid treatment is stopped, then casting ensues (Wislocki *et al* 1947). However, the fact that plasma levels of testosterone are low during velvet antler growth indicates that there is no direct velvet antler growth stimulating capacity of testosterone (Fennessy and Suttie 1985). At various times a variety of hormones including prolactin (Mirarchi *et al* 1978) luteinising hormone (West and Nordon 1976) and growth hormone (Bubenik *et al* 1975) have been considered as antler stimulating hormones. However, none of these hormones have direct cartilage growth promoting activity and in view of the fact that the developing velvet antler is composed of cartilage, it is hardly surprising that they have been found lacking as antler stimulating hormones.

### i. Growth factors

At Invermay we measured insulin-like growth factor 1 (IGF1) in the plasma of young red deer stags (Suttie *et al* 1989). This hormone was known from studies in other animals to be a cartilage growth stimulator. Not only did we show that IGF1 increased during the spring and summer compared with the autumn and winter, but there was a very close correlation between IGF1 and velvet antler growth (Figure 1). We concluded that this growth factor, which was known to stimulate cartilage growth, was probably responsible for stimulating velvet antler growth. However, we could not make a definitive statement because the growth factor might have been just correlated with velvet antler growth or a consequence of it. To definitively state that IGF1 stimulated growth, we would need to show receptors in the tissue for IGF1 and also show that isolated antler cells in culture would grow if treated with IGF1.

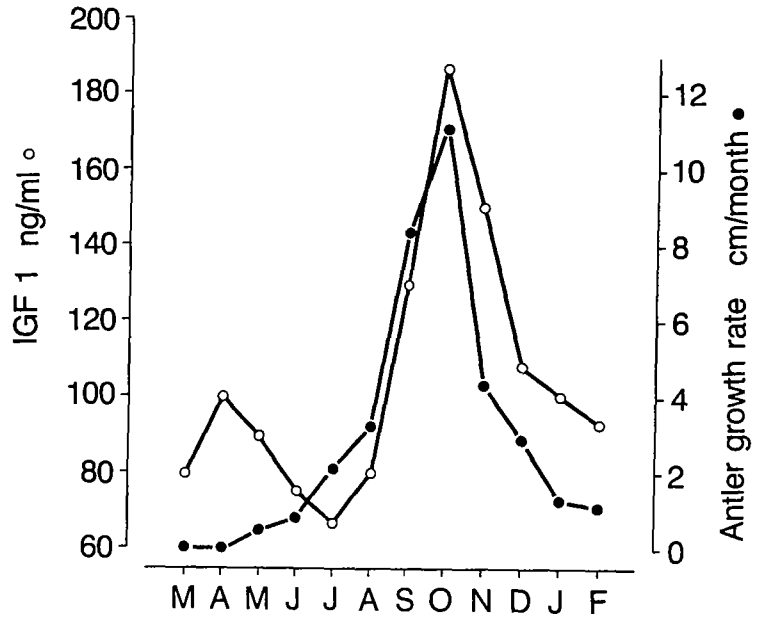


**Figure 1.** Plasma IGF1 and antler growth rate during a calendar year in 6 red deer stags. The stags were 4 months old at the start of the study.

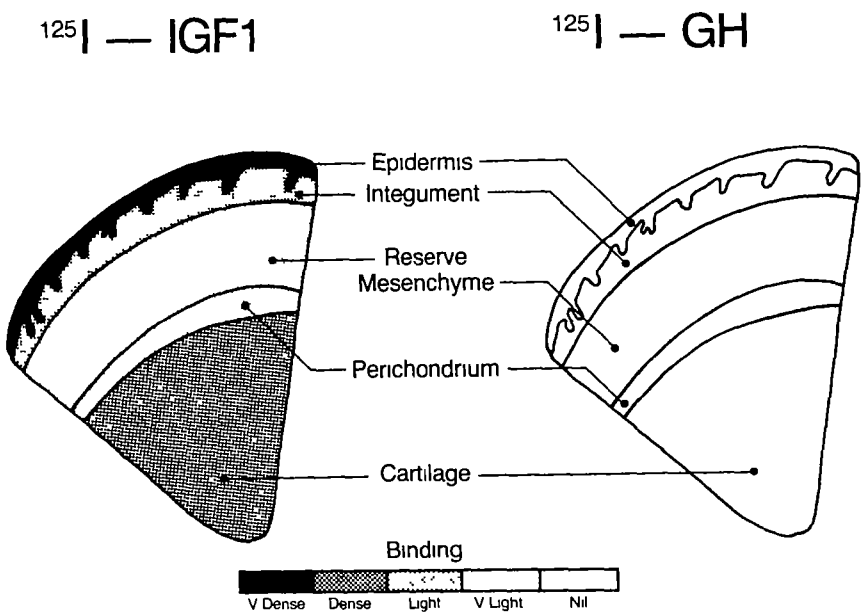


**Figure 2.** Diagrammatic representations of cryostat sections of antler tip labelled with either $^{123}$I-labelled IGF1 or growth hormone. The extent of specific binding is shown by the intensity of shading. Note that there is no specific binding by GH to this region of the antler.

We have taken cryostat sections of velvet antler tip, treated them with $I^{125}$-labelled IGF1 or $I^{125}$-labelled growth hormone (GH), (GH from the pituitary is known to cause the release of IGF1 from peripheral tissues such as the liver) and exposed a radiographic film to them (autoradiography) (Elliot *et al* In subm). Areas where the receptors are located in the tissue are shown clearly as dots on the autoradiograph, using this technique. The results (Figure 2) showed that there was no specific GH binding in the antler but IGF1 bound specifically particularly in the epidermis and the cartilage. When binding in 2 separate zones of the cartilage was studied, more binding was found in the zone of hypertrophy than the zone of proliferation (Figure 3). This means that IGF1 is mainly associated with cartilage matrix synthesis rather than cell division.

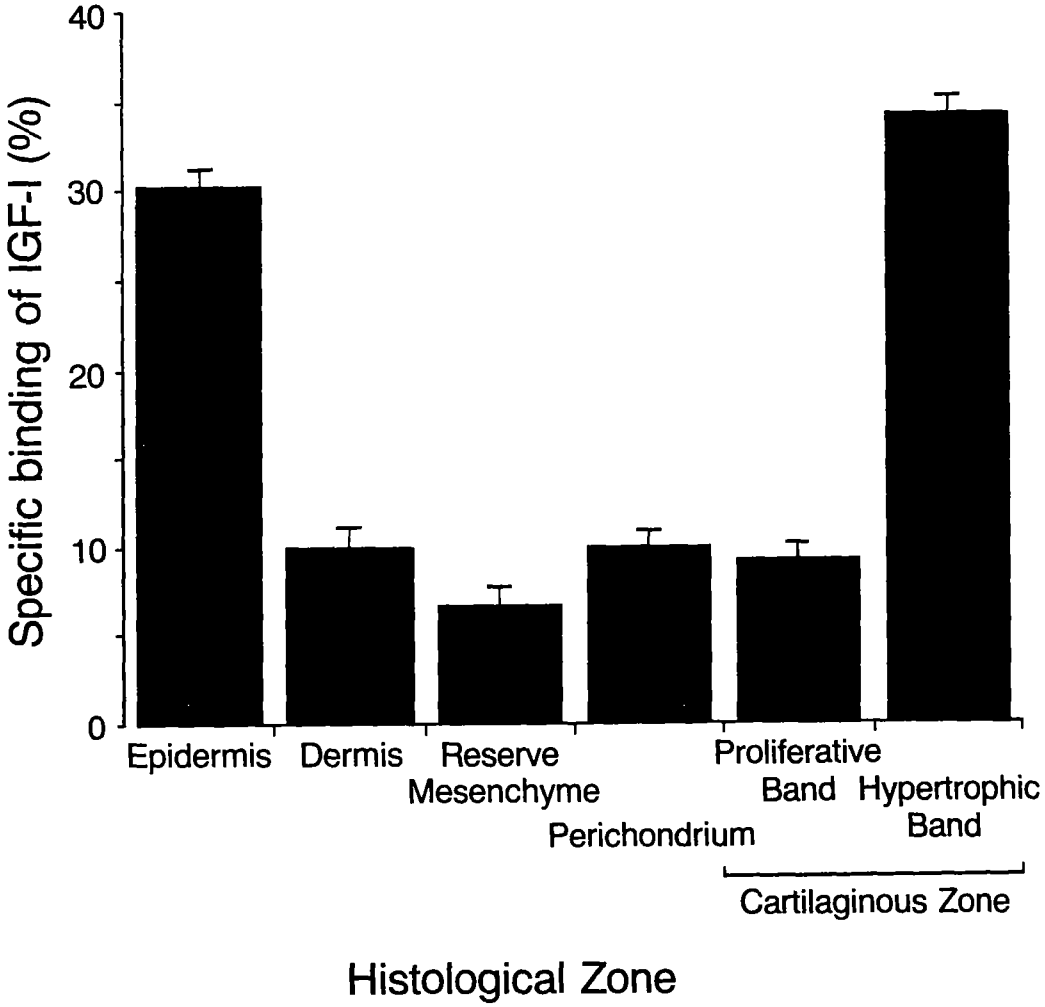


**Figure 3.** Quantitative assessment of specific binding of IGF1 to histologic zones in the antler tip.

In a further series of studies, the antler tip was homogenised and specific binding measured in the homogenates using $I^{125}$-IGF1 and $I^{125}$-GH. Because we did not expect GH to bind in the antler, a homogenate of deer liver was also tested as a positive control. The binding data show that there was clear specific binding of IGF1 in the antler but although GH bound to receptors in the liver, there was no such binding to the antler (Figure 4). Scatchard analysis (Figure 5) revealed an affinity constant of 22.4 nmol/l for the interaction of IGF1 with the type 1 IGF1 receptor, with a capacity of 15.8 pmol/l per 100 mg initial tissue weight.

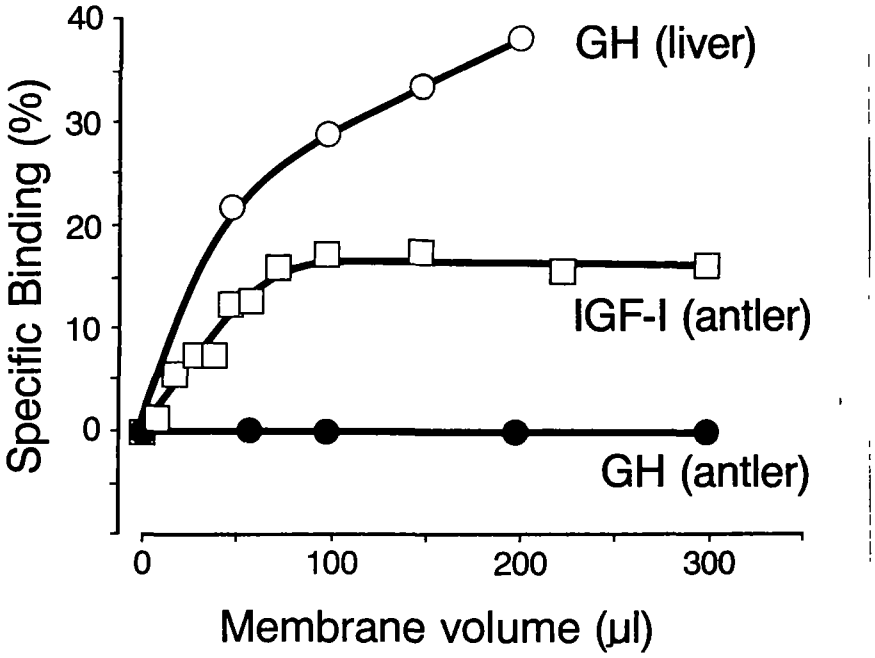


**Figure 4.** Specific binding of GH and IGF1 to deer liver or antler homogenate.

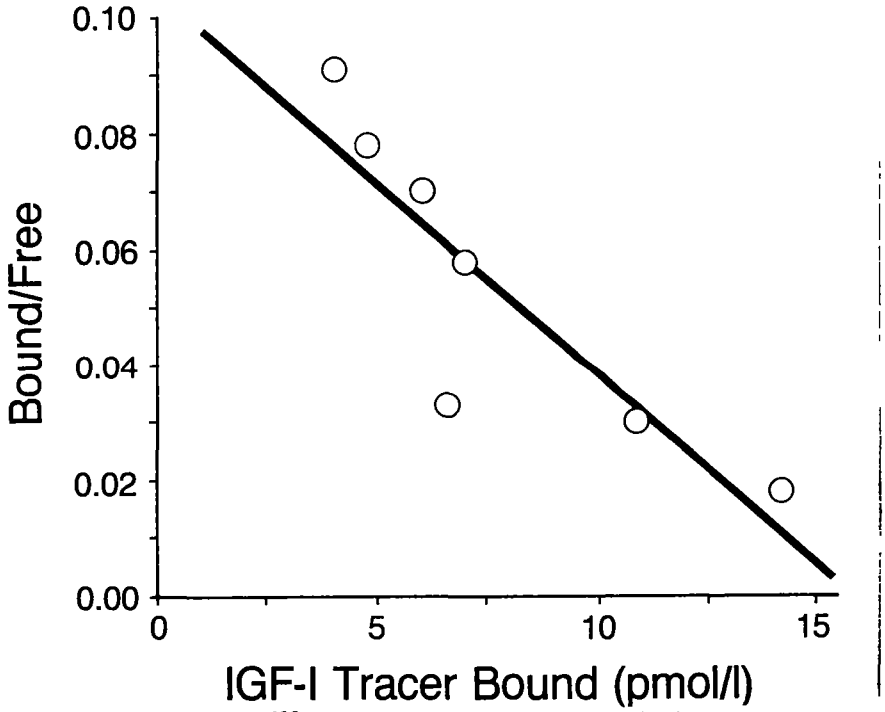


**Figure 5.** Scatchard plot of $^{125}$I-labelled IGF1 in antler tip homogenates.

These data tend to confirm earlier speculation that IGF1 is involved with velvet antler growth and further demonstrate that its primary involvement is in cartilage matrix development. It appears that the IGF1 may not be produced in the antler as GH, its normal trophic agent in other tissues apparently lacks receptors in the antler. It also means that GH itself, can have no antler growth promoting effects.

ii. *In vitro* study of velvet antler growth

Deer antler has become recognised as an excellent model for a number of biomedical studies of bone growth and mineralisation and the influence of the endocrine system. Growing antler tissue is very similar to developing skeletal bone. However, there are numerous advantages

in using the antler as a model to study bone growth: such as its external position, access to blood vessels and nerves, lack of muscles, fast growth rate, massive mineralisation process, the deciduous nature of antler growth, and the extreme sensitivity to the endocrine system.

In recent years, many growth factors have been isolated from extracts of bone matrix and/or bone organ culture media, including transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), skeletal growth factor (SGF), acidic and basic fibroblast growth factor (FGF) and $\beta_2$-microglobulin. Of these factors, the IGF and TGF-β series are particularly important because they stimulate the proliferation of both pre-osteoblast and more mature bone cells, while basic fibroblast growth factor (bFGF) mostly stimulate chondrocyte DNA and inhibit sulfated proteoglycan synthesis and Type II Collagen mRNA.

Methods

*Primary Culture of velvet antler*: Samples of undifferentiated zone (fibroblast) and osseous zone (osteoblast) tissues were taken from the tips of a growing velvet antler when they were 50 cm long. Samples were incubated with collagenase (200 unit/ml) in 45% $BGJ_b$, media (Fitten-Jackson modification) 45% $F_{12}$ nutrient media (nutrient mixture $F_{12}$ Ham), 10% Foetal bovine serum (FBS) at 36.5°C for 4 hours. The cell suspension was rinsed once with the media and cultured. Cells were grown to confluence and were passaged with 0.1% trypsin in calcium and magnesium-free PBSS (Phosphate buffered saline Solution A). Cells were subsequently passaged when needed using a 1:10 dilution ratio.

*Alkaline phosphatase (AIP) assay*: AIP activity was determined using PNPP (P-nitrophenyl phosphate) as a substrate in a reaction mixture containing 30 mM PNPP, 150 mM carbonate buffer and 1 mM MgCl 2 (PH 10.3). The absorbence was determined by spectrophotometer at 495 nm.

*Cytochemical staining of AIP activity*: Cells were detached and rinsed with BSS then they were spread on slides. They were dried either at room temperature or 37°C. Slides were fixed in (25 ml citrate, 65 ml acetone and 8 ml 37% formaldehyde) for 30 seconds and stained in diazonium for 15 min at room temperature. They were washed with deionized water and dried.

*Cloning*: Cloning was used to overcome the heterogeneity of the cells in primary and isolate a pure cell strain.

The cells were diluted and single cells were seeded in multiwell plates and cultured in media (40% $BGJ_b$, 40% $F_{12}$ nutrient, 20% FBS). The media was changed every 7 days. Colonies were transplanted in separate flasks after 3 weeks, using viokase (10 times diluted) to detach the cells as viokase is more gentle to the cells than trypsin.

Results

Primary cultures have been prepared from fibroblasts and osteoblasts. They have been through 13 passages and both have faster growth rates at earlier stages (Fig. 6). There is no difference in AIP activity between these two types of cells histochemically and biochemically.

This fast growth rate at an early stage *could* be because of growth factors either as autocrine controls or perhaps the cells are unable to continue to grow at the same rate because they lack endocrine or paracrine support. The cloned fibroblast and osteoblast cell lines are now ready

for *in vitro* work to determine the roles of growth factors in antler cell division and differentiation.

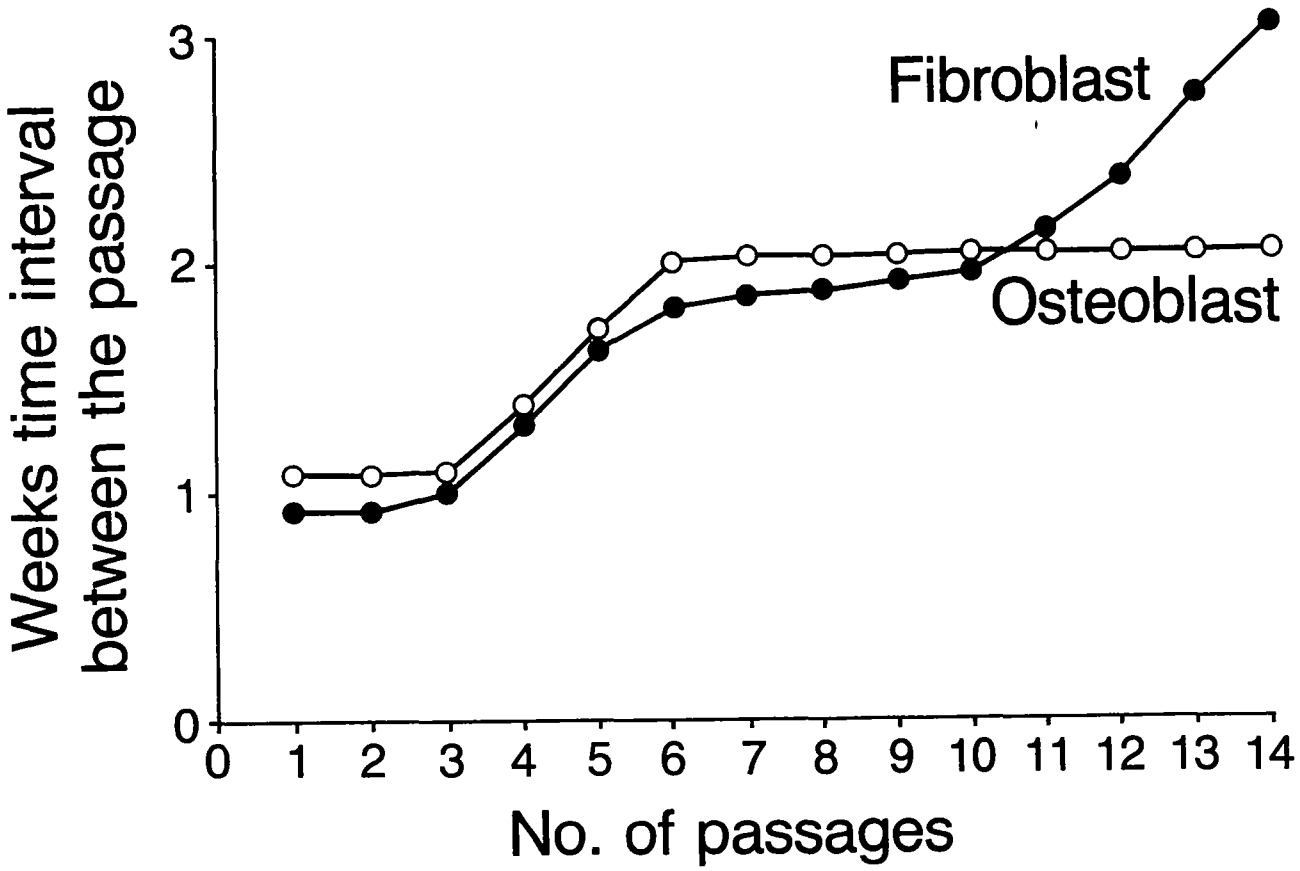


**Figure 6.** Growth rate of cultured antler tip fibroblasts and osteoblasts.

**PART B Serial radiography, with hormone measurements, of antler casting and cleaning**

Although radiography offers a non-invasive, repeatable, atraumatic technique of studying antler growth and the antler cycle, the application has been limited by those studying antler growth. Brown *et al* (1978) studied the relationship between serum androgen concentrations and relative density changes in antlers and long bones using radiography. However, he neither published antler radiographs during the antler cleaning process nor studied the mechanism of antler casting. We have studied the endocrine (luteinising hormone, (LH) and testosterone) changes between castration and antler casting, testosterone implantation and antler cleaning and testosterone withdrawal and antler casting in young red deer stags. On each occasion blood sampling took place a radiograph was made of the antler-pedicle junction.

Two stags were castrated every 3 months for one year (n=8) under Xylazine anaesthesia and xylocaine local analgesia at the winter and summer solstices and the spring and autumn equinox. The first pair of stags were 16 months old when castrated and the last pair were 25

months old. The animals were anaesthetised with Xylazine every 3-4 days between castration and antler casting and were injected intravenously with 20 ng/kg liveweight luteinising hormone releasing hormone (LHRH) and blood samples were taken at 0, 10, 40, 75 and 120 minutes after the injection. All samples were analysed for LH and testosterone by radio-immunoassay. On each occasion a radiograph of the antler pedicle junction was made with a portable x-ray machine using 60 pKV, 12 mA for 0.3 secs at a focus to film distance of 10 cm (which was critically adhered to) on Min-R film (Eastman Kodak Ltd).

Six months after antler casting one of each pair (n=4) was implanted with 180 cm of silastic testosterone implants. Previous studies had indicated that they should maintain a plasma testosterone concentration of 2-4 ng/ml in castrate stags. LHRH challenges and radiography were carried out at weekly intervals until antler cleaning was complete.

One month after antler cleaning the testosterone implants were removed and LHRH challenges with radiography were carried out at 3-4 daily intervals as above until antler casting.

On each occasion an intact stag was blood sampled and x-rayed, as a control.

LH and testosterone data for 6 of the 8 stags which were castrated and for one intact stag are shown in Figure 7. As two of the stags were already in velvet when castrated, no induced casting could occur. In all the stags plasma testosterone was either very low or was rapidly reduced by castration. LH levels increased after castration, presumably due to the lack of the steroid negative feedback signal. The pair castrated in March (peak rut aged 16 months) took 21 days from castration to antler casting, those castrated in June took 14 days and those castrated in September 10 days. Thus there is a trend for a decrease in the timing of casting following castration with season such that as the normal time of casting (spring) approaches the time taken for the withdrawal of gonadal steroids to permit casting decreases. The intact stag, number 40, had a progressively decreasing LH response to GnRH as time progressed. This was reflected in a greatly diminished release of testosterone. From 4 days prior to antler casting the stag essentially produced neither LH nor testosterone. Thus at casting the stag resembled a castrate in having no circulating testosterone. The series of radiographs (Figure 8) shows the progressive changes in stag number 6 from castration to casting. Within 3 days of castration a thin line is visible below the coronet indicating bone resorption. After that at day 7 clear lines can be seen at the dorsal and frontal aspects of the antler pedicle junction. These lines, which are two dimensional representations of a band around the circumference of the antler pedicle junction, become larger and eventually, 4 days before casting, appear to completely separate the dead antler bone from the living pedicle bone.

The responses of the stags to the testosterone implants varied (Figure 9). In 2 cases, numbers 37 and 6, levels of testosterone were high enough to feedback negatively on LH and thus reduce the responses to LHRH. This was not so for the remaining 2 stags; the reasons for this are unclear. What is clear is that the implants in all cases caused antler cleaning after 21-35 days of treatment. The intact stag had testosterone levels elevated naturally for about the same length of time prior to cleaning. Taken together these results indicate that testosterone responses must be about 4 ng/ml for 3-5 weeks to permit antler cleaning.

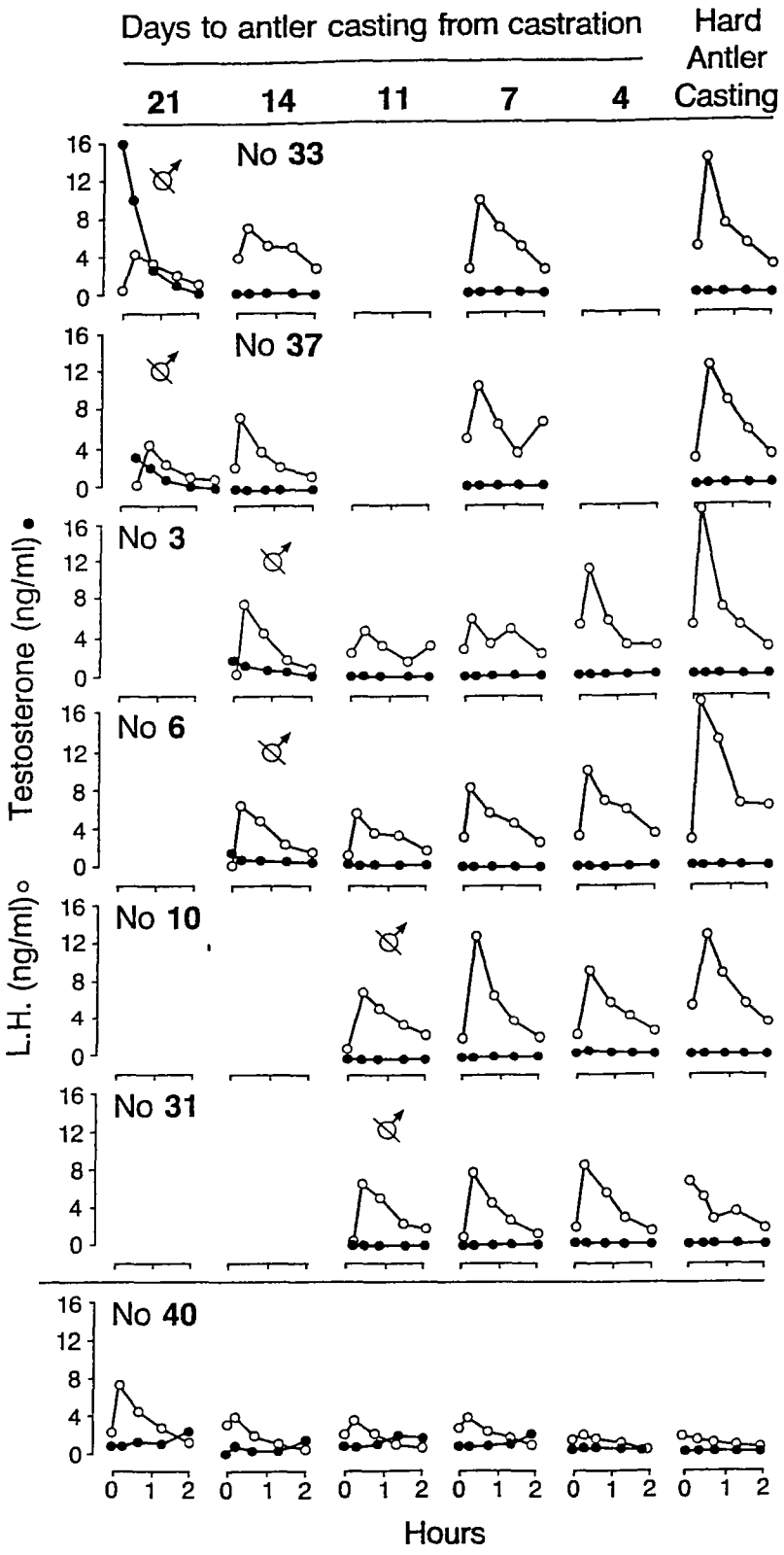


**Figure 7.** LH and testosterone responses to LHRH from castration to the casting of the hard antler in 6 castrate stags. Stags were blood sampled for 2 hours after the LHRH. The remaining 2 stags which were castrated were in velvet when castrated. Data from one intact stag (No. 40) is included for comparison.

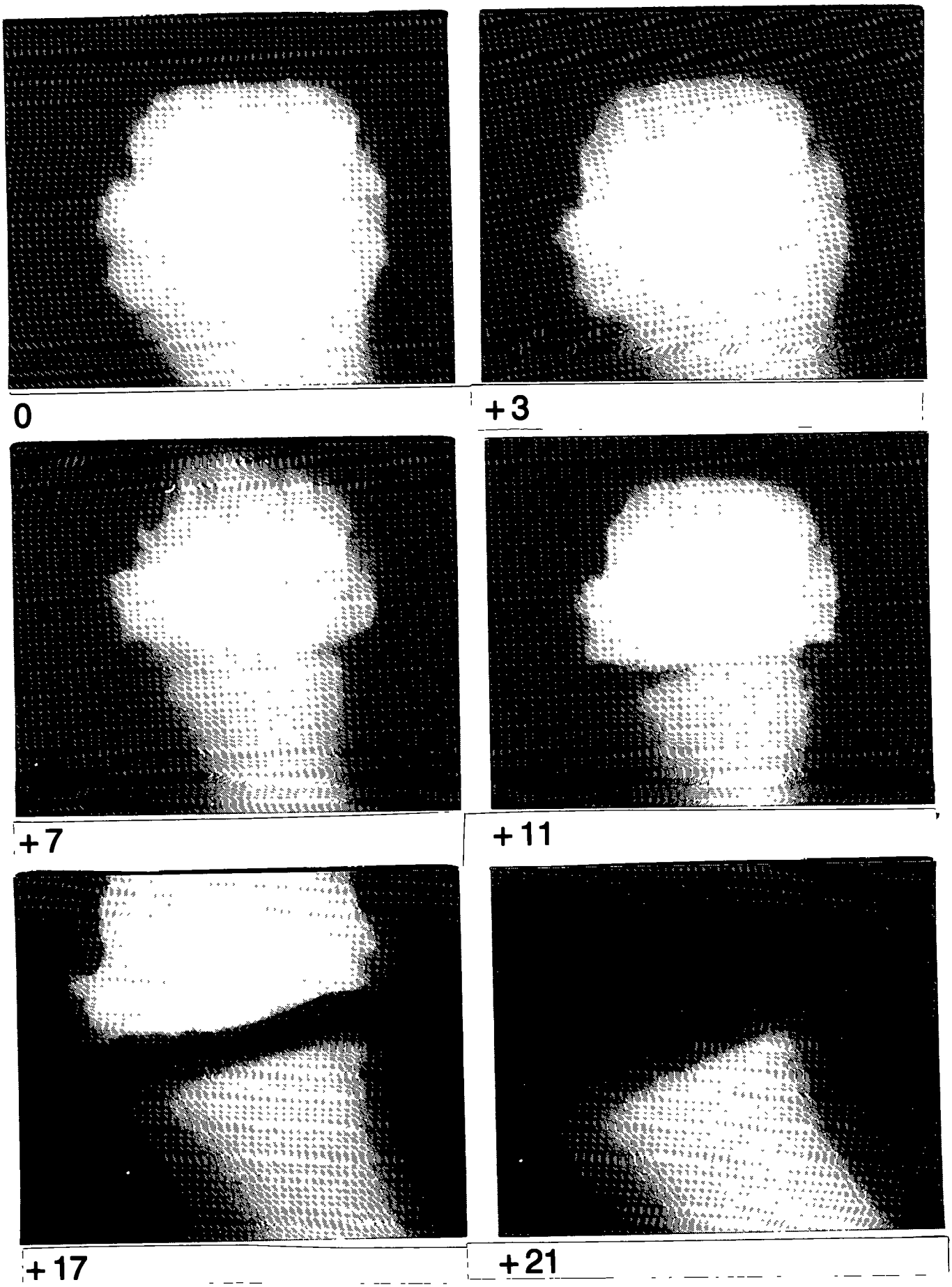


**Figure 8.** Serial radiographs taken (0, 3, 7, 11, 17 and 21 days) between castration and had antler casting in one typical stag.

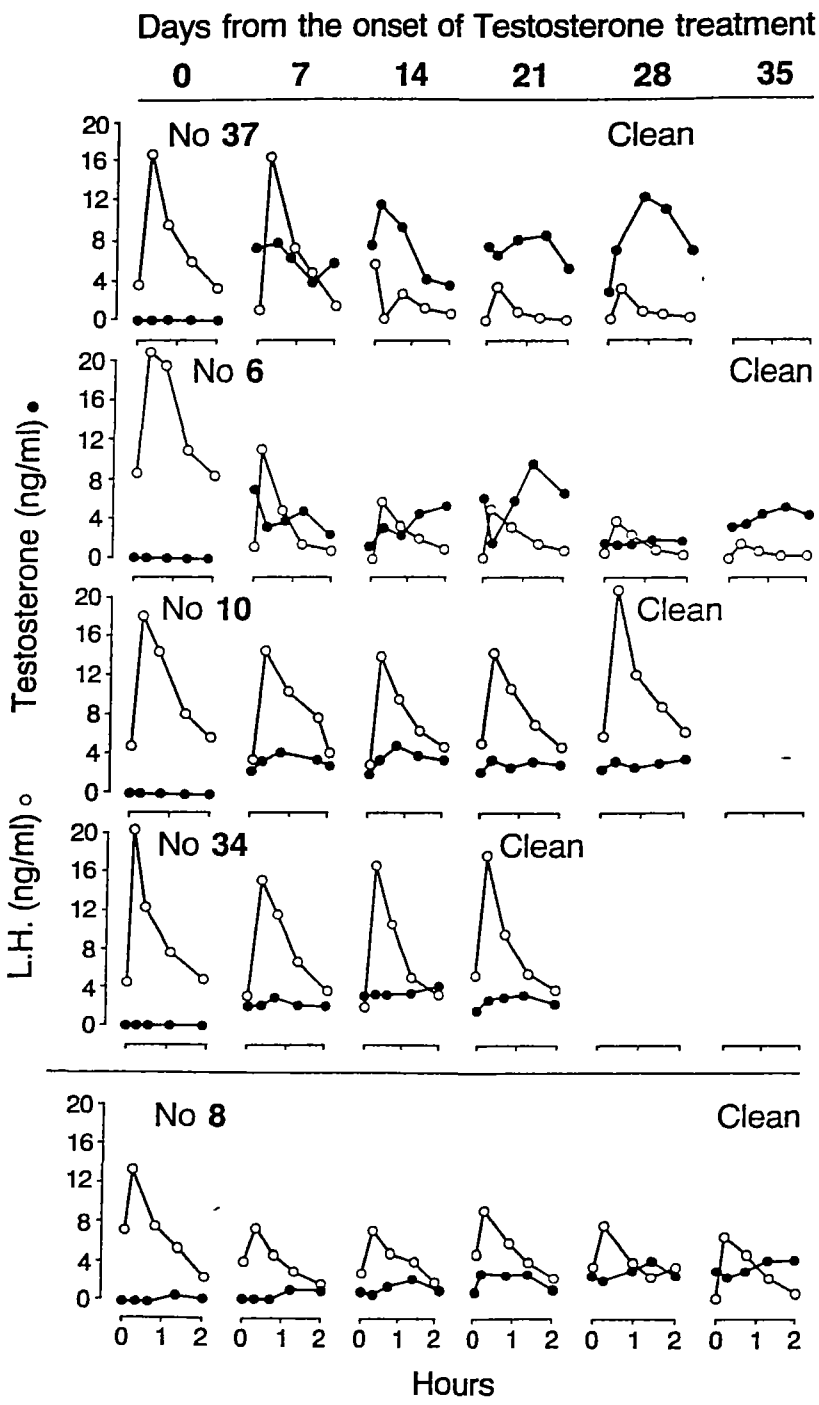


**Figure 9.** LH responses to LHRH in 4 castrate stags treated with silastic testosterone implants to induce antler cleaning. Because the plasma concentration of testosterone are not trophically related to the LH, there is no predictable pattern of testosterone plasma level. One intact stag (No. 8) is included as a control. The animals were blood sampled for 2 hours after the LHRH injection.

The serial radiographs (Figure 10) taken at weekly intervals from the onset of testosterone treatment to antler cleaning indicate a progressive thickening of the bone and a development of "pearling" around the base of the antler. Brown *et al* (1978) found that the relative bone density increased 4 fold between velvet antler to hard clean antler in whitetailed deer. Interestingly serum androgen levels rose from 2 ng/ml to 3-7 ng/ml over this period.

After the testosterone implants were removed the stags cast their antlers 21 (numbers 37, 10 and 34) and 24 (number 6) days later (Figure 11). The radiograph series from Stag 6 indicates a similar pattern to the series following castration, except that the separation between the antler and the pedicle is cone shaped (Figure 12). This is normal in castrate deer whose antlers have cleaned after testosterone treatment (Bubenik 1990). It probably indicates that the junction between living and dead bone is not at the coronet at the time of antler cleaning, but may "migrate" downwards so that at the natural time of casting it is in the typical position at or slightly below the coronet.

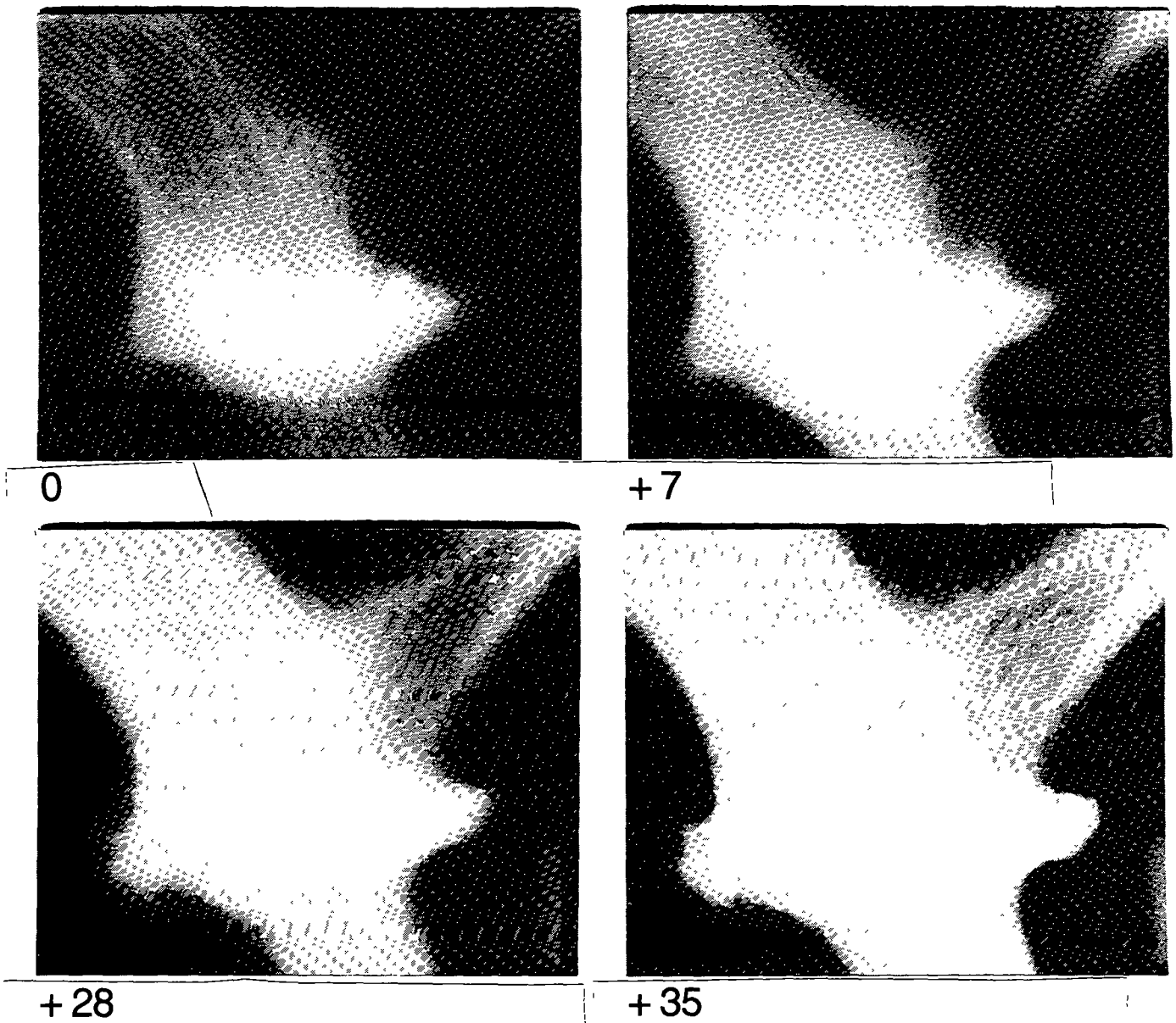


**Figure 10.** Serial radiographs of antler of a castrate stag (N0. 6) taken at 0, 7, 28 and 35 days after treatment with testosterone until antler cleaning.

## General discussion

The novel approaches have confirmed the notion that a growth factor known to stimulate bone and cartilage growth (IGF) has potency in the antler. We are now ready to carry out the *in vitro* work necessary to precisely define the roles of the different growth factors in determining antler cell growth, differentiation and division.

The radiography provides a unique anatomical picture of antler autotomy and has confirmed the role of steroids in influencing the timing of this event.

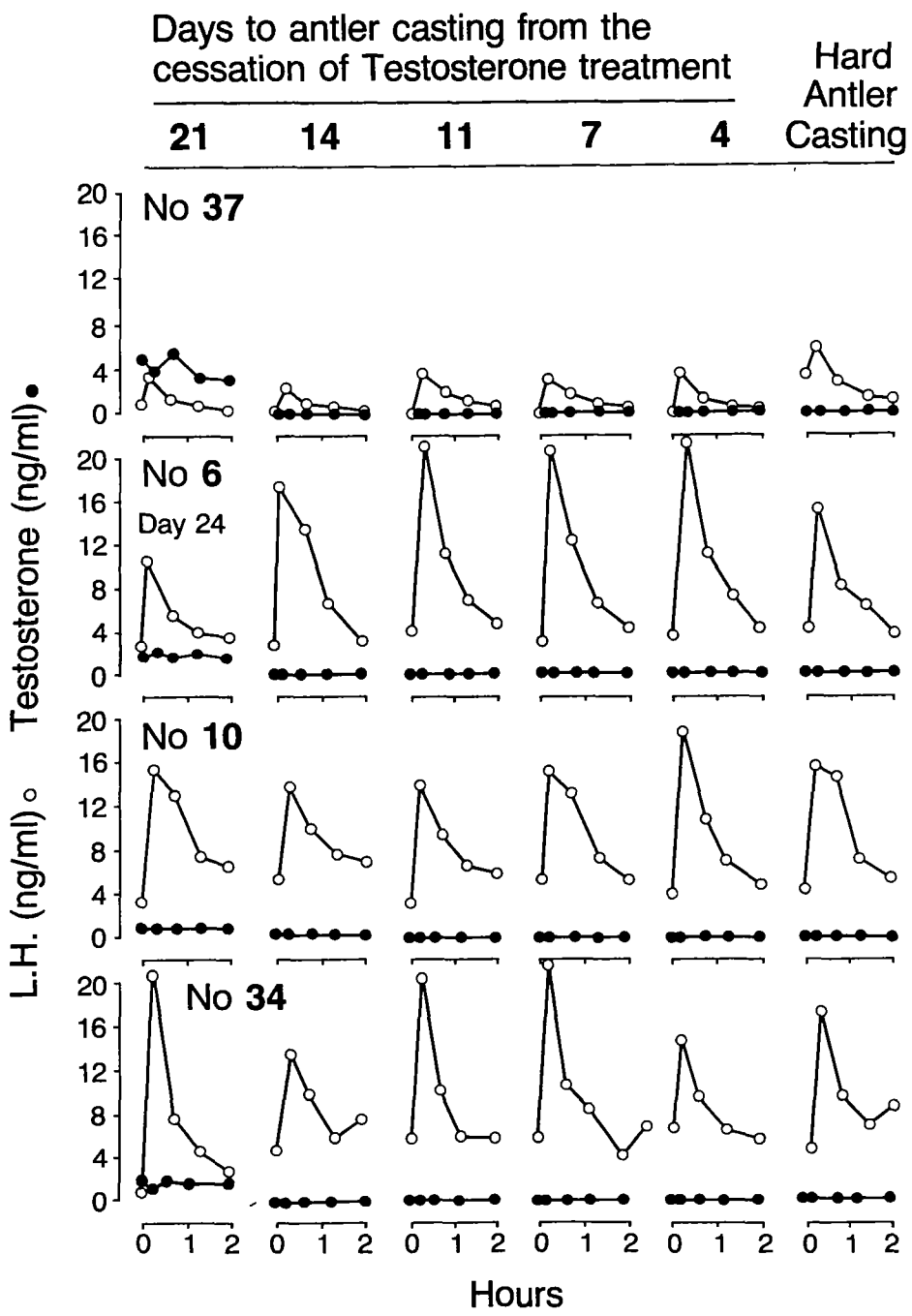


**Figure 11.** LH responses to LHRH for 2 hours after injection of LHRHand testosterone levels in 4 castrate stags from withdrawal of testosterone implants to antler casting.

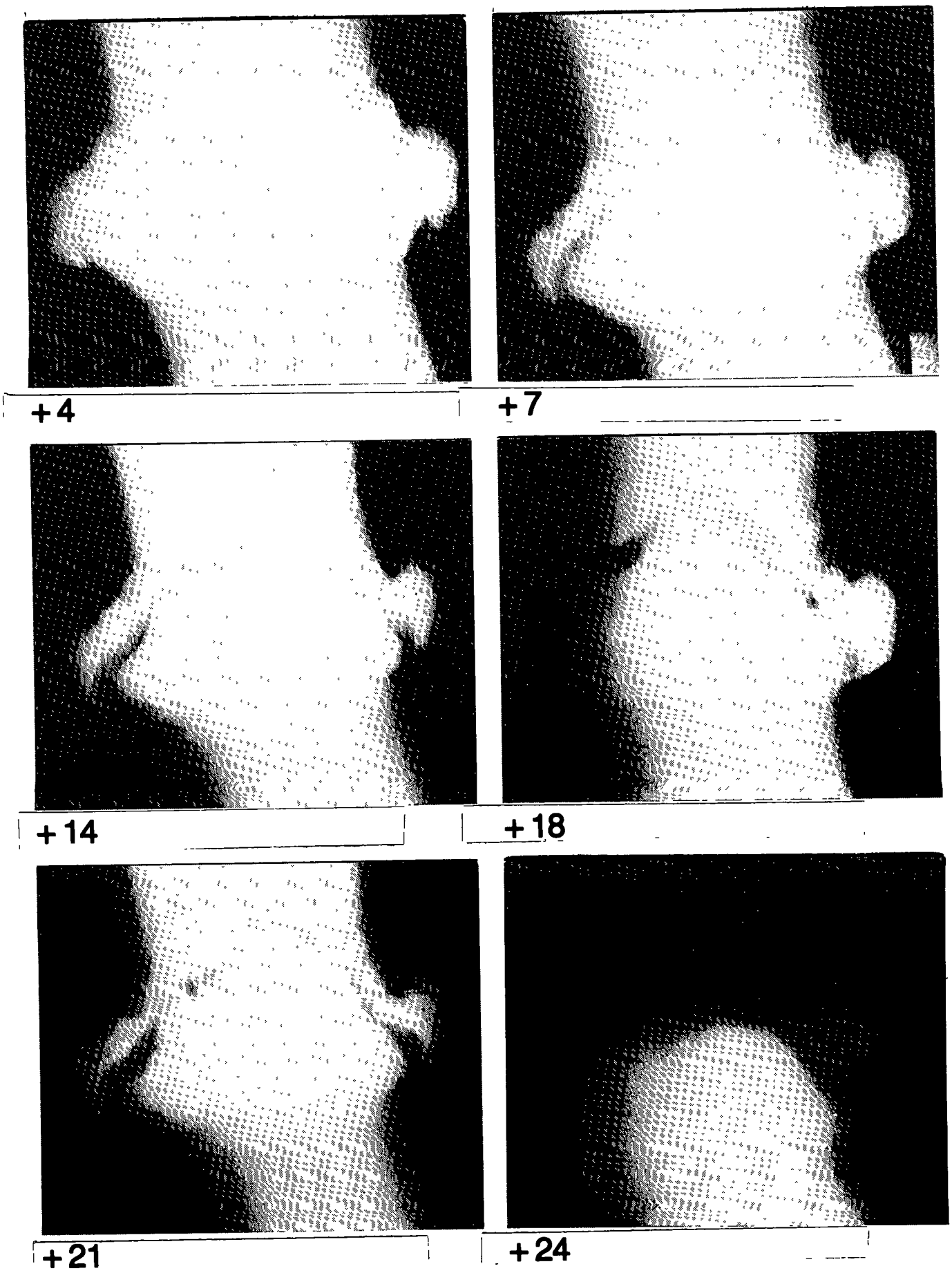


**Figure 12.** Serial radiographs of a castrate stag (No. 6) taken 4, 7, 14, 18, 21 and 24 days after the withdrawal of testosterone treatment.

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