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First Belgian Workshop on Animal Endocrinology

With the success of the third International Conference on Farm Animal Endocrinology - The Somatotropic Axis (3° ICFAE) (Brussels, 7–10 December 1998; abstracts published in BASE, 1998, vol. 2, special issue), the Belgian members of the committee have decided to continue the experience by organizing an annual Belgian Workshop on Animal Endocrinology. The first edition of it was held at Gembloux on the 13th of October 1999. The scope of this 1999 edition workshop and the forthcoming ones is to focus on endocrine functioning of productive and/or reproductive processes in domestic animals including bovine, ovine, porcine, poultry, equine, canine, murine, etc. Examples of more specifically defined topics are:
- Growth including embryonic development;
- Lactation, including sustained production and mammary gland development;
- Metabolism, including endocrine control of physiological functions sustaining production;
- Reproduction including follicle functioning, gestation, puberty and birth control.

A major goal of the workshop is to give young scientists and PhD students the opportunity to present their own research in a forum of experts in several areas of basic and applied research in animal endocrinology. Indeed, as stated by Prof. Decuypere at the conclusion of the 3° ICFAE (*Domestic Anim. Endocrinol.* 1999 **17**, p. 329–330): "The combination of fundamental and applied sciences, of different approaches to well-defined problems, cannot or rarely is realized by a single research group or institution. The relatively new phenomenon in research is the attempt to develop a fruitful and constructive approach to current problems through collaboration between fundamental and applied sciences and scientists". The committee hopes that launching these annual workshops will be the beginning of a new adventure to promote endocrinology research. Finally, we greatly appreciate the financial support of Biocode Biotechnology and invite you at the 2nd Belgian Workshop on Animal Endocrinology in Leuven (October 2000).

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Abstracts

RECEPTORS AS SCREENING TOOLS IN THE DETECTION OF HORMONES. APPLICATIONS IN THE CONTROL OF MEAT PRODUCTION

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Keywords. Hormone, anabolic, alucocorticoid, ß-adrenergic agonists, receptor.

The control of the use of growth promoters in animal production has become an important issue. Illegal treatments are applied using various substances: steroid derivatives with sex hormone activities (androgen, estrogen or progestagen), glucocorticoids, or ß-adrenergic agonists. Generally, the molecular structure of the anabolizing agent is not known. As a consequence when screening methods like immunoassays are used for the control, their high specificity generally limits the number of controlled substances to 5 or 6. Compared to the number of different susbtances (more than 50) that have been identified in cocktails or injection sites, it is clear that another screening strategy was needed. In order to respond to a demand for methods able to detect known and newly synthesized steroids or ß2-agonists, we are interested in setting up receptor assays allowing the detection of residues of substances used as meat production enhancers. These analytical methods have an advantage over immunoassays as screening methods: they are multianalyte.

Two types of systems are in development:

1. Receptor assays based on a competition between a labelled hormone and hormone-like substances, present in the sample extract, for the binding to

hormone receptors. We attempt to produce these hormone receptors, or at least their hormone binding region, by genetic engineering. This type of assay is under development for substances with estrogenic, androgenic, progestagen, glucocorticoid or ß2-adrenergic activities.

2. Steroid hormones act through a receptor found in cytosol of many mammalian cells. This specific interaction provides a second means for developing assay methods. Enzymes (luciferase: LUC) or fluorescent proteins (Green Fluorescent Protein: GFP) are induced through the binding of the steroid to the receptor.

1. Material and methods

Binding inhibition receptor assays. Expression of recombinant receptors was performed in *E. coli*. The hormone binding domain of the human estrogen receptor (ER) was fused in frame to the C-terminus domain of the glutathion-S-transferase gene (GST) and the soluble recombinant protein was recovered by centrifugation after bacterial lysis. The human ß2-adrenergic receptors used in this study are expressed in the plasma membrane of *E. Coli* (gift of D. Strosberg, Paris).

Steroid hormone responsive DNA elements (SRE) were coupled to a promoter followed by the gene coding for LUC and GFP. The assay uses the receptor present in a cell to mediate the interaction between the steroid hormone and SRE. The luciferase enzyme assay produces light that can be very sensitively detected. GFP producing cells can be easily observed under a fluorescence microscope.

2. Results and discussion

Expression of receptors. For estrogen receptors, co-expression of a fusion protein with a chaperone lead to the production of up to 30 mg of soluble, active protein per liter of culture. For the other steroid hormones work is in progress to optimize the production of soluble proteins able to bind with high affinity substances with androgenic, progestagenic or glucocorticoid activities. Optimized culture conditions lead to the production of 350 pmoles of ß2-adrenergic receptors per liter of bacterial culture.

Binding studies. The relative binding of various estrogens was assessed by measuring the binding inhibition of tritiated 17ß-estradiol to ER caused by increasing concentrations of various estrogenic susbtances. For ß-agonists, their relative binding compared to clenbuterol was determined using radioactive iodocyanopindolol.

Cell based assays. Two types of plasmid-transfected cells, T47D and, were used for the detection of glucosteroids, androgens or progestagens. All these binding receptor assays and cell bioassays are sensitive enough for controlling hormone-contaminated samples with a limit of detection of the order of 1 ppb. Samples found positive in the screening should be confirmed by mass spectrometry coupled to gas or liquid chromatography.

EFFECT OF ALPHA-LACTALBUMIN AND BETA-LACTOGLOBULIN ON THE SUPEROXIDE ANION PRODUCTION OF BOVINE MILK NEUTROPHILS

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Keywords. Milk, neutrophil, alpha-lactalbumin, beta-lactoglobulin, chemiluminescence.

As a result of respiratory burst activation, milk neutrophils produce reactive oxygen species (ROS) which can play a crucial role in the killing of endocytosed microorganisms. Neutrophil function is known to be impaired following diapedesis through the blood/milk barrier. In addition, there is some evidence for a decreased of the concentration of casein, alpha-lactalbumin (α-L) and beta-lactoglobulin (β-L) in milk during mammary gland infection. The relative contributions of α-L and β-L to the ROS production of milk neutrophils have not yet been investigated. Neutrophils were isolated from seven healthy high yielding Holstein cows during mid-lactation. α-L and β-L concentrations of 0.5, 1, 1.5 and 2 mg/ml and 3, 4, 5, and 6 mg/ml, were used, respectively. We investigated the influence of different concentrations of α-L and β-L on the production of ROS both by a phorbol 12-myristate 13-acetate (PMA)-induced luminol enhanced chemiluminescence (CL) and by a superoxide dismutase-inhibitale reduction of ferric cytochrome c assay. For α-L, the CL response of neutrophils increased by 10% at 1 and 1.5 mg/ml (< 0.05) and peaked at 2 mg/ml with 18% above the control value (< 0.01). For β-L, the CL response increased at 5 mg/ml (< 0.05) by 11% and was maximal at 6 mg/ml (< 0.001) with 23% above the control value. The increase of superoxide anion production was concentration-dependent ($< .05$) for both α-L and β-L. These results indicate that the impaired function of neutrophils in milk can not be attributed to α-L and β-L. On the contrary, elevated levels of whey proteins such as α-L and β-L could lead to stimulation of neutrophil function. Further studies are required to investigate the immunomodulatory

effects of whey proteins. (This study has been supported by the Belgian Ministry of Agriculture and by the Ministry of Culture and Higher Education of Iran).

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SALIVARY CORTISOL RESPONSES TO WEANING IN SOWS

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Keywords. Pigs, weaning, salivary cortisol, stress coping, welfare.

The public concern about intrinsic quality of pork, but also about how it is produced, expanded enormously. In order to address this concern and to regain the confidence of the consumer, a thoroughgoing search for improvement of the management techniques with a view to welfare is indispensable. The current weaning procedure includes a number of stressful events for the sow: a withheld meal, abrupt separation from the piglets, change in housing and an increasing tension in the udder. In present study, the salivary cortisol response to abrupt weaning in sows was evaluated in order to estimate its impact on the sow's welfare and to explore the possible influence of parity and genetic background.

A total of 48 purebred sows of seven different genetic lines were used. These 2nd to 5th parity sows were weaned after a lactation period of 4 weeks by removing the sows out of the farrowing crate and moving them to another room into individual stalls. Feed was withheld on the day of weaning. Saliva was sampled before and after the weaning procedure by allowing the sows to chew on a cotton bud, mounted on a plastic rod. After 30 seconds, the rod was withdrawn and the bud was centrifuged at 2500 G in order to drive the saliva out of the bud. Afterwards, the saliva was stored at –18 °C until analysis. Salivary cortisol concentrations were measured by using a commercial enzyme-immunoassay kit (ENDAB, Biogenesis Ltd, UK). All samples were analysed in duplicate. The intra- and inter-assay variation coefficients were 13.57, res. 14.07 %. The results were analysed in an analysis of variance according to the General Linear Models (SAS Institute Inc., 1994).

The population mean of the rise in salivary cortisol in percentage terms was 121.1 (S.E. = 120.4) and statistically significant (paired t-test, $P < .0001$). The large variation indicates big individual differences. The span between the pre- and postweaning samples (11 to 60 min.) had no effect on the results. Parity and genetic line had no influence on the rise in salivary cortisol. The absence of an association between the change in salivary cortisol level and the parity suggests a lack in habituation or desensitisation to this procedure in multiparous sows. A follow up of individual sows during a number of consecutive parities could confirm this. Whatsoever, the present results corroborate earlier studies based on behavioural (de Passillé, Robert, 1989) and plasma cortisol (Tsuma *et al.*, 1995) responses that the current weaning procedure is a stressful event not only for the piglets, but also for the sows. Besides, saliva collection proofed to be an easy, stressfree way to determine the biologically active free cortisol.

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MODULATION OF THE BIOLOGICAL ACTION OF BOVINE GROWTH HORMONE BY PASSIVE IMMUNIZATION IN HYPOPHYSECTOMISED RAT

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Keywords. Immunopotentialisation, bovine growth hormone, hypophysectomised rat, mechanism of modulation, monoclonal antibodies.

It has been stated that the administration of correctly chosen anti-bovine growth hormone (bGH) monoclonal antibodies (MAbs) enables potentialisation of the biological action of GH in *in vivo* models, such as hypophysectomised rats (Massart *et al.*, 1993), pigs and cattle (Beattie, Holder, 1994). The results obtained in two experiments carried out on Wistar hypophysectomised female rats are presented here. In the course of these experiments, the following treatments have been tested: saline buffer (control), bGH (100 µg/rat) alone or complexed with 400 µg/rat of anti-bGH monoclonal (6B1, 2H4) or polyclonal (R99) antibodies. An anti-GH-binding protein (GHBP) MAb (9A8) has also been tested in the presence of bGH and/or with 150 µg/rat of recombinant GHBP-maltose binding protein. During the first experiment, the animals were undernourished and lost weight, which made them non-receptive towards the treatment. Underfeeding may have induced a reduction of the hepatic GH receptors number (Maiter *et al.*, 1988). By reducing the stress applied to the animals (supplementation with thyroxine and cortisone, liquid supplementation with glucose), we managed to maintain the body weight during the second experiment. Various endocrine parameters were analysed: serum insulin-like growth factor-I (IGF-I) measured by radioimmunoassay, IGF-binding protein-3 (IGFBP-3) and GHBP concentrations studied by Western ligand blot, and liver IGF-I total RNA determined by dot blot. The expected potentialisator effect varied greatly depending on the applied treatments. The GH-supplemented group reacted in increasing its body weight, IGF-I total RNA, and serum IGF-I and IGFBP-3 concentrations. As for the 2H4 and R99 antibodies, they did not act as potentialisators of GH action. The GHBP+bGH group did not react on the first day of the treatment, but well on the second one, probably because of a feedback working on GHBP which enabled bGH to act on its receptors. This group took weight on the second day. As to the 6B1+bGH group, it presented a potentialisation of the GH action with serum IGF-I and IGFBP-3 concentrations that were significantly higher than the other groups. In this study, IGF-I total RNA was however not affected by the potentialisator antibody 6B1. In fact, only the GH-treated group presented an increase in its IGF-I total RNA. Using the results obtained with the 6B1+bGH and bGH groups, the mechanism by which the anti-bGH MAbs potentialisate GH action might be explained by the following hypothesis: the potentialisator complex might lead to regular activation of the cellular GH receptors on several occasions. In this case, such a phenomenon could favour the regular production of small quantities of total RNA. The bGH would be protected by the anti-bGH antibody which would guide it repeatedly towards the GH receptors. Consequently, a serum IGF-I accumulation might have taken place inducing likewise a considerable body weight gain of the rats treated with 6B1+bGH.

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AUTOMATED ŒSTRUS DETECTION IN INDIVIDUALLY HOUSED SOWS BY MONITORING BODY ACTIVITY AND SALIVA PROGESTERONE

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Keywords. Sows, œstrus detection, infrared sensor, body activity, transrectal echography, ovulation.

A good œstrus detection influenced the amount of produced piglets per sow per year positively. Œstrus detection and insemination is very intensive and takes almost 30% of the time of the stockman. An amelioration of œstrus detection would improve productivity of primiparous sows and would reduce the number of sows replaced in relation to reproduction problems.

Sows (207) were housed individually within commercial stalls (2 m × 0.65 m) situated in an naturally ventilated service house with a partially slatted concrete floor. Environmental temperature was within the thermal neutral zone. Sows were fed twice a day and water was available ad libitum.

An infrared sensor, as described by Freson *et al.* (1998) and Geers *et al.* (1995), was placed above the front of the sow to monitor the body activity continuously. Œstrus was surveilled by transrectal echography, progesterone concentration in saliva and standing responses before the boar and the herdman. Changes in activity and progesterone concentration were analysed in relation to œstrus and ovulation. Data were corrected for the animal effect and for the sampling period.

Mean activity increased during the oestrus period (P<.01). Activities measured during the night (24:00) and in the morning (08:00) could inform about ovulation, as measured by transrectal echography. Œstrus, as shown by the standing responses of the sow, could be predicted by the increased activities observed between 09:30 and 22:00. Progesterone concentration was below 1ng/ml during ovulation time, and an increase was found five days after ovulation.

An algorithm was optimised to permit the classification of the sows "in heat" or "not in heat". The data were analysed by logistic regression and activity during the day after weaning, sampling period and genetic line were taken into account. The sensitivity of the test expresses the amount of sows classified as "in heat" on the total amount of sows which were in œstrus (true positive animals). The specificity of the test indicates the animals tested "not in heat" on the totality of sows not in œstrus (true negative animals). An increase in the threshold value decreases the number of false positive animals, but increased the number of animals in the false negative group. The oestrus and anoestrus period could be separated. Œstrus, as detected with standing responses, can be predicted with a sensitivity of 88.5% in combination with a specificity of 76.5% after the introduction of mean activity, day and sampling period and genetic line in the algorithm. The progesterone concentration could not be used to predict ovulation time. These results show the possibilities for automated œstrus detection, but the accuracy must still be improved for practical use. As standard, only transrectal echography can be used, while saliva concentrations of progesterone were not accurate. (Funding was provided by the IWT, Belgium. R. Geers is a member of the ASAS and was supported by the FWO-Flanders (Belgium)).

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PLASMA IGF–I CONCENTRATION IN COMBINATION WITH BLOOD GAS MEASUREMENTS IN BLOOD CAN PREDICT THE GROWTH CAPACITY OF PIGS

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Keywords. Swine, growth capacity, plasma, IGF-I, bloodgas.

Growth capacity of pigs can be predicted by growth models, using body composition at the start and chemical composition of the diet as input parameters, while feed conversion, growth parameters and body composition are output parameters (Van der Peet-Schwering *et al.*, 1994).

It is expected that the reliability of the predicted results on an individual level may increase by introducing endocrinological and metabolic parameters in the model.

Piglets originating from two genetic lines (60 homozygous halothane positive and 60 homozygous halothane negative) were weighed at 20 kg, 40 kg, 60 kg, 80 kg and around 100 kg. Body composition was determined at each body weight by Dual Energy X-Ray Absorptiometry (DEXA; DPX-L, Pediatric Software Lunar, USA) and by SKG-II at slaughter. At a body weight of 20 kg intravenous catheters were inserted into the ear veins of the piglets. After a recovery period of at least 24 hours, venous blood was sampled 2 times in fasting conditions and 4 times after refeeding to measure pH, pCO_2, pO_2, HCO_3- and IGF-I. Plasma IGF-I levels were log transformed to alleviate non-normality. Statistical analysis was performed using the Proc Mixed procedure (SAS, 1994). Covariance structures (SIMPLE, CS, UN, AR(1) or TOEP(1)) were compared using Schwarz Bayesian Criterion (SBC). Within these Mixed procedures, each pair of least square means was tested with a *t*-test (SAS, 1994).

Halothane positive pigs needed 16 days longer (110 vs. 94 days from birth; P <.0001) to obtain a body weight of 40 kg compared to their halothane negative counterparts. This differences in daily growth rate remained significant until slaughter. Body weight, bone mass content and fat mass remained lower in halothane positive pigs, but lean mass was higher at a body weight of 40 kg (38.1 kg vs. 36.6 kg; P <.02). These differences remained significant until the end of the experimental period.

AR(1) was used as covariance structure in the analysis of IGF-I, pH, pO_2, pCO_2 and HCO_3- levels in the blood of the piglets. Median levels of IGF-1 were higher in halothane negative than in halothane positive pigs (76.6 ng/ml vs. 25.5 ng/ml; P <.0001) and differed significantly at each sampling point.

Both pH and pO_2 were significantly lower in halothane positive than in halothane negative piglets (pH: 7.378 vs.7.393; pO_2: 38.6 vs. 42.2 mm Hg; P <.0001).

Plasma IGF-I levels were positively correlated with daily weight gain and increases in fat mass, while pO_2 and pH levels in halothane positive pigs were negatively linked with the higher capacity for lean meat deposition, which implies a larger impact on the homeostasis of the pigs in stress conditions. These conclusions are in accordance with the finding that faster growing broiler chickens have a stronger susceptibility to ascites in a challenging environment.

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SULFATION OF THYROID HORMONE IN CHICKEN

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Keywords. Thyroid hormone, sulfation, characterization, chicken, ontogeny.

Next to deiodination, glucuronidation and sulfation of the phenolic hydroxyl group are other important pathways of iodothyronine metabolism. Although in general conjugation serves to facilitate the urinary and biliary excretion of lipophilic substances by increasing their water solubility, sulfation of thyroid hormones has also another important function.

The interaction between sulfation and deiodination has already been described in several mammalian tissues and species. Sulfation of the phenolic hydroxyl group blocks the deiodination by the type II deiodinase (D2) and by the type III deiodinase (D3). This could be important in situations where type I deiodinase (D1) activity is low or impaired and the sulfate conjugates are not degraded. Sulfation, therefore, protects the tissues against an excess of active thyroid hormone. Under the same conditions, it can be used as a reservoir from which active hormone is released by sulfatases when and where the active thyroid hormone is required.

This interaction between sulfation and deiodination was an important reason for us to explore the sulfation activity in the chicken. After an initial characterization of the sulfotransferases we have studied the changes in sulfation during chicken ontogeny. Sulfation activity in tissue cytosolic fractions was determined by a method developed by Kaptein *et al.* using radioiodinated 3,3'-diiodothyronine (3,3'-T2) as the substrate, cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as the sulfate donor and Sephadex LH-20 minicolumns for separation of the reaction products.

Sulfation assays demonstrate the presence of sulfotransferases in liver, kidney and brain cytosol in one-day-old chicken. Similar to the situation in mammals, the enzyme(s) shows a substrate preference for 3,3'-T2 > 3,5,3'-triiodothyronine (T3) and 3,3',5'-triiodothyronine (rT3) > thyroxine (T4). There is a linear increase of sulfation of 3,3'-T2 when we use protein concentrations ranging from 0,1 to 0,5 mg/ml. In our sulfation assay, however, we need to take into account the presence of some deiodinating activity in the cytosol. When using rT3 and T3 as substrates we find a high amount of free I^-, which is a product of deiodination. The T2 which is formed in this way, can in turn be sulfated thereby resulting in an overestimation of sulfation activity. A higher sucrose concentration or centrifugational velocity, however, prove not to be enough to remove all traces of iodothyronine deiodinases from the cytosol.

Measurements of sulfation during ontogeny from 16-day-old embryonic chickens (E16) to one-day-old posthatch chicks (C1) demonstrate that sulfation activity varies independently in liver, kidney en brain, suggesting that the activity is regulated in a tissue specific manner. Liver activity decreases from E16 to E19 followed by an increase from E20 to C1. Sulfation activity in the kidney increases slowly while there is a more pronounced rise in the activity in the brain from E16 towards hatching.

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EXPRESSION OF THE THYROTROPIN-RELEASING HORMONE RECEPTOR AND THE CORTICOTROPIN-RELEASING HORMONE RECEPTOR IN THE PITUITARY GLAND OF THE CHICKEN (*GALLUS GALLUS*)

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Keywords. Chicken, thyrotrophs, *in situ* hybridization, TRH-R mRNA, CRH-R mRNA.

In the chicken (*Gallus gallus*) thyrotropin (TSH) secretion by a discrete cell population in the pituitary, called thyrotrophs, is controlled by several hypothalamic hormones. Classically thyrotropin-releasing hormone (TRH) is considered to be the main releasing factor of TSH, but recent evidence suggests that in

fowl, especially corticotropin-releasing hormone (CRH) is a potent stimulator of TSH secretion (Geris *et al.*, 1996). Recent cloning of the hypophyseal TRH- and CRH-receptors allows us to see if both receptor proteins are expressed by the chicken thyrotrophs in order to verify whether a direct or a paracrine action is occurring. To visualize the expression of the receptors in the thyrotrophs, we applied the technique of *in situ* hybridization.

Pituitaries were collected from adult female layer chickens (Hisex, Euribrid, Belgium) and tissue sections were made with a cryostat. Three antisense oligonucleotide probes based on the base sequence of the chicken β-subunit of TSH (TSH-b), the chicken TRH-receptor (TRH-R) and the chicken CRH-receptor (CRH-R) were synthetized (Eurogentec, Belgium). The probes were labeled with ^{35}S-dATP or ^{33}P-dATP, using the Dupont Nensorb™ 20 nucleic acid purification cartridge (NEN, Boston USA). A hybridization cocktail (containing formamide, SSC, BSA, polyvinylpyrrolidone, lauroylsarcosine, phosphate buffer, dextrane sulphate and Ficoll 400) was used to accomplish in situ hybridization between the tissue's mRNAs and the added radioactive probes. After an overnight incubation and four washes with 1× SSC, tissue sections were dried and the hybridization signal was detected with an autoradiographic Hyperfilm-β max (Amersham, UK). As a control condition, in situ hybridization was performed with the sense probe or after prior treatment with RNase A.

Best results were observed when the *in situ* hybridization was performed at 37°C and washing occurred at 42°C. Thyrotrophs (i.e. cells that showed a positive signal after hybridization with the antisense TSH-b probe) were only seen in the cephalic lobe of the pituitary, conform to earlier immunocytochemical studies (Iwasawa *et al.*, 1998). The same part of the pituitary contained the mRNAs for the CRH-R. Since not only thyrotrophs, but also corticotrophs (producing corticotropin) are located in this lobe, and CRH-R mRNA is likely to be found in the corticotrophs, further research is required to elaborate the cellular localization of this receptor. TRH-R mRNA on the other hand, was mainly found in the caudal lobe, presumably in the somatotrophs that produce growth hormone. This observation supports the idea of Kühn *et al.* (1993), that TRH has no thyrotropic function in the adult chicken.

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ONTOGENETIC STUDY OF THE ACTIVITY AND EXPRESSION OF IODOTHYRONINE DEIODINASES DURING CHICKEN EMBRYOGENESIS

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Keywords. Chicken, embryogenesis, iodothyronine deiodinases, regulation.

Because iodothyronine deiodinases (D) play a crucial role in thyroid hormone metabolism, we studied the ontogeny of these enzymes in chicken embryos. There are three deiodinases, type I (D1), type II (D2) and type III (D3) which are defined by the reactions they catalyze, their substrate preference and their sensitivity to inhibitors. D1 catalyzes inner and outer ring deiodination (IRD, ORD), D2 always catalyzes ORD and D3 only catalyzes IRD reactions. In our study samples were taken from plasma, telencephalon, cerebellum, diencephalon, optic lobes, brainstem, pituitary, thyroid gland, spleen, heart, gut, lung, liver, ovaria, testes, bursa Fabricii, skin, muscle and kidney of chickens on day 16 of the incubation (E16) until 1 day after hatching (C1). Half of the chickens on E20 was in the pipping stage (E20IP), while the other half was non-pipping (E20NP). Enzyme activities were determined by specific in vitro kinetic tests. Northern blotting with D1 and D3 cRNA and D2 cDNA probes was executed to investigate deiodinase mRNA expression. Plasma T4, T3, GH and TSH concentrations were determined by radioimmunoassays.

High *in vitro* D1 activity was found in liver, gut and kidney, intermediary D1 activity in lung, testes and ovaria and low D1 activity in bursa Fabricii, spleen and pituitary. We only detected D2 activity in different parts of the brain, where it was present in equal quantities. *In vitro* D3 activity, on the other hand, was present in every tissue studied. It was high in gonads and liver, intermediary in kidney, brain and bursa Fabricii and low in gut, lung, spleen, pituitary, skin, muscle and heart. Almost all of these *in vitro* activities were confirmed by measuring the respective deiodinase mRNAs in each organ. Comparison of in vitro activity and mRNA expression patterns allows some speculation about the level of regulation of the deiodinases. In gut D1 activity and expression show a similar increase up to hatching. This suggests that the regulation of D1 in gut takes place at the pretranslational level. D2 expression in brain increases at the end of incubation and drops after hatching. In diencephalon, cerebellum and brainstem we detect the same mRNA pattern which indicates that D2 is regulated pretranslationally. D3 apparently is regulated at the posttranslational level in bursa

Fabricii and in skin because activity and mRNA show different patterns. In liver and gut, on the contrary, D3 seems to be regulated pretranslationally since both D3 activity and mRNA increase up to hatching.

The presence of D3 in every organ studied suggests an important role for this enzyme during chicken ontogeny. D1 and particularly D2 are expressed in far less organs. In most organs where D3 is present abundantly, we detect very little D1 or D2 activity. On the other hand there seems to be active D1 or D2 in tissues that possess very little D3 activity. Liver forms an exception, because it is the only tissue where both D1 and D3 are abundantly expressed. Iodothyronine deiodinases seem to be regulated in a tissue specific way, since both pretranslational and posttranslational regulation seem to take place in different tissues. We did not find any differences in the expression and regulation of the deiodinases between male and female animals except in gonads.

IDENTIFICATION OF ANIMALS TREATED WITH BOVINE SOMATOTROPIN: A NEW NON-ISOTOPIC DETECTION SYSTEM FOR BST.

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Keywords. bST, identification, ECL, milk.

The treatment with recombinant bovine somatotropin (rbST or recombinant bovine growth hormone) is used to improve the dairy cows production. Biotechnology like this is able to alter the confidence of consumers in agricultural noble products, but also to disrupt commercial exchanges and interfer with animals genetic selection programs. Our objective was to quantify bST in plasma as well as in milk, in order to provide a practical screening test to identify treated animals. In *Exp. 1*, Belgian White Blue heifers were injected (n=6) with bST preparation (500 mg rbST, *Posilac*®, Monsanto™) or not injected (n=4, control) every two weeks during four months. The second experimental group (*Exp. 2*) contained injected (n=10) or not injected (n=11, control) Holstein cows. First treatment (500 mg rbST, *Posilac*®, Monsanto™) began on day 70 *postpartum*, and animals were treated every two weeks during seven months. A new technology, the electrochemiluminescent (ECL) detection system, has been adapted for bST quantification in plasma and milk. We have developed a sandwich-type assay. First, a monoclonal mouse antibody (6B1, 25 ng/ml) against rbST (Monsanto™) is labelled to an *N*-hydroxysuccinimide ester of ruthenium (II) tris-bipyrimidine chelate (TAG-NHS ester, Igen Inc.). Second, a biotinylated monoclonal mouse anti-rbST (Monsanto™) antibody (8D5, 25 ng/ml) is used, which react with streptavidin pre-coated on magnetic beads (Dynabeads® M-280, Dynal®). The major characteristics of the ECL method are the absence of radioactive materials, the stability of TAG-labelled reagents and a detection limit as low as 5 pg bST/ml. The typical standard curve has an effective range of 12,25 pg/ml to 3,125 ng/ml. The slope, y-intercept, R^2 and ED_{50} ratio averaged 1.451, 1.804, 0.992 and 83.41, when incubation of samples with antibodies requires 24 hours at room temperature. Intra- and inter-assay coefficients of variation were less than 5%, and we demonstrated parallelism when using 10 to 50 µl of sample. Results obtained for plasma bST concentrations with the ECL 6B1-8D5 system were correlated with values provided by RIA ($r = 0,81$) and ELISA ($r = 0,71$) methods. During bST treatment, plasma bST levels increased according to the rythm of injections. Highly significant differences ($P < .01$) were observed after the first injection (*Exp. 1*) between treated and control animals, until the end of the trial. Animal response was maximum 5 to 9 days after the first treatment, reaching 33.08 ± 3.11 ng/ml and 18.38 ± 5.77 ng/ml, respectively. During the trial, control animals averaged 0.26 ± 0.19 ng/ml to 5.43 ± 2.23 ng/ml. In *Exp. 2*, bST levels increased significantly ($P < .05$) after the second treatment (4.73 ± 1.28 ng/ml), when results for control animals have slightly varied during the trial (2.51 ± 0.79 ng/ml to 3.29 ± 1.50 ng/ml). In our study, the ECL method was the only technique usable for determination of bST concentrations in milk. Milk bST were significantly higher ($P < .05$) after the first injection, and remained significantly higher ($P < .05$) than in the control group during the trial, reaching concentrations above 0.42 ± 0.12 ng/ml (*Exp. 2*). Values for control group varied between 0.25 ± 0.03 ng/ml and 0.33 ± 0.10 ng/ml.

In conclusion, our study shows that, in experimental conditions, it is possible to screen bST treated animals by measuring hormone itself in plasma or milk during the treatment. This ECL-based assay provides a reliable alternative to RIA and ELISA, characterized by a high antibodies specificity and an improved assay sensitivity. In addition to a noted stability of TAG-labelled reagents, this method offers the opportunity to eliminate the use of radioisotopes.

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MODULATION OF GROWTH HORMONE ACTION BY ACTIVE IMMUNIZATION IN DAIRY COWS

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Keywords. Growth hormone, milk production, cow, immunization, immunopotentialization.

In previous studies, we characterized a monoclonal antibody (MAb) raised against natural bovine growth hormone (bGH) which potentiated bGH activity when injected into hypophysectomized rats. An economically important though not universally accepted application domain of recombinant bGH or somatotropin (bST) is the treatment of dairy cows during the mid and late lactation period, increasing milk yield with an average of 5 kg/day. In our experiment, we tested whether immunomodulation could be valid alternative for or supplementation to bST treatment. Seventy days postpartum, 30 Holstein cows were assigned to three groups following a block design. A first group received any treatment (control group). A second group was treated with bST (Posilac® (Monsanto)) slow-release preparations administered every two weeks. A third group was immunized with a vaccine construction, comprising of the GH epitope recognized by the previously characterized MAb, the active component of Freund's complete adjuvant (muramyl-dipeptide) and the T-epitope of ovalbumin; this peptide was emulsified with an adjuvant (Montanide ISA50) and injected on week 0, 2, 4, 9 and 19 of the experimental period. Blood samples were taken on the vaccination days before treatment; plasma was obtained and stored at –20 °C. Using an ELISA with direct coating of the antigen, the production of antibodies directed against ovalbumin and the peptide construction was detected in the immunized group, indicating that the immunized cows had developed a good immune response against the peptide construction. Our ELISA for anti-bGH antibodies however did not permit to discriminate between the different experimental groups, suggesting that the immune response was not maximally effective from functional point of view. Milk production in immunized cows attained an intermediary level between control and bST-treated cows, largely due to a sustained milk yield in the immunized group between week 7 and 15, in contrast to decreasing milk production in the bST-treated group. Total plasma IGF-I concentration, as quantified by RIA, was strongly increased in the bST-group, showed a moderate increase from the 9th week of experiment onwards for the control group, but stayed low in the immunized group. Plasma IGFBP-3 concentrations were analyzed using Western ligand blot. A noted IGFBP-3 increase in the bST-treated group was observed, whereas the immunized animals tended to have lower IGFBP-3 levels than the control group. Plasma IGFBP-2 levels as evaluated by RIA were from the 3rd vaccination onwards increased in the immunized group and transiently decreased for the bST-group. In general, the immunized animals showed opposite endocrine responses as compared to bST-treated cows, although the treatments indirectly or directly augment GH action, eliciting questions about the working mechanism of the potentiating antibodies raised in the immunized animals. Several hypotheses have been proposed from studies in rat models, including increased bGH systemic half-life, enhanced or prolonged binding of bGH to somatogenic receptors and increased selectivity for certain cell types (1,2). In conclusion, immunomodulation of GH action seems a promising alternative for bST-treatment but requires more research to improve the vaccine construction, immunization protocol and biological action, and to exclude adverse immune effects, in order to obtain a maximal economical benefit.

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THE ANORECTIC EFFECT OF JOJOBA MEAL AND SIMMONDSIN AND THE REPERCUSSION ON PLASMA LEPTIN LEVELS IN DOGS

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Keywords. Simmondsin, jojoba meal, leptin, satiety, dogs.

Leptin is a protein hormone mainly synthesized in and secreted from white adipose tissue in proportion to both adipocyte size and number. It is a potential afferent signal of fat stores and as such an important element of the long-term acting energy intake and expenditure regulating system. A variety of experimental work proves that leptin influences the activity of or the sensitivity for several hormones and neurotransmitters as cholecystokinin (CCK), neuropeptide Y (NPY),

insulin or glucocorticoids. Each of these molecules is at its turn important in the control of food intake and energy expenditure. Mostly mice and rats have been used as a model to study the working mechanism and physiological function of leptin in the body. Nothing is known about the role of leptin in weight regulation or in the long-term acting satiety system in dogs.

Jojoba meal is the protein rich rest product that remains after the extraction of jojoba oil from the seeds of the jojoba plant (*Simmondsia chinensis*). Simmondsin is a cyano-glycoside present in jojoba meal at about 5% and exerts a food intake reducing effect in rats through the interference with the CCK system. A food intake experiment was set up to study the possible anorectic effect of jojoba meal (JO) and simmondsin (SIM) in dogs. Eighteen adult Labrador dogs were divided into 6 groups of 3 animals each and were adapted for 4 weeks to *ad libitum* feeding. Five different kinds of experimental food were prepared: a standard control dog food (C) supplemented with (i) jojoba meal in 3 different concentrations: 0.12 (JO12), 0.24 (JO24) and 0.36 (JO36) % simmondsin equivalent, and with (ii) pure simmondsin in concentrations of 0.12 (SIM12) and 0.36 (SIM36) %. Each experimental food was given ad libitum for 5 days to each group of dogs in a Latin square design and with 2 days of rest between successive treatments. The food intake was recorded daily and expressed as a percentage of the control food intake of the same group. On day 5 the dogs were weighed and a blood sample was taken from a vein of the forelimb. Blood leptin levels were measured using a commercially available multispecies radioimmunoassay kit (Linco Research Inc.) that was validated for dog plasma leptin measurements.

JO24 and JO36 caused a food intake reduction of about 25% while SIM36 exerted a decline of the voluntary food intake of approximately 20%. When feeding JO12 or SIM12, no significant food intake reduction could be observed. After 4 weeks of adaptation to *ad libitum* feeding an average body weight increase of 0.8 kg/dog was observed. After C, JO12, SIM12 and SIM36 treatment a minor gain of body weight was recorded. JO24 and JO36 caused a loss of body mass of 0.3 kg/dog and 1.0 kg/dog respectively, due to an average daily food intake below the maintenance level. A gain of body weight was significantly correlated with an increase of the plasma leptin concentration and, analogous, a weight loss corresponded in a statistically significant way with a decline in the plasma leptin level ($r = 0.35$, $P < .05$). However, the average difference in leptin concentration after all 5-day treatment periods was very small with a large SEM (0.062 ± 0.12 ng/ml). Therefore, it is questionable whether a 5-day test period is long enough to provoke a significant alteration of the blood leptin concentration. It also remains speculative whether the observed alterations in plasma leptin levels are sufficient to elicit an effect on food intake or energy expenditure.

PAGs MOLECULES AS MARKERS OF PREGNANCY IN THE BOVINE: COMPARISON OF CONCENTRATIONS OBTAINED BY USING THREE DIFFERENT ANTISERA

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Keywords. Bovinae, pregnancy, pregnancy associated glycoprotein.

The mammalian placenta is a source of several proteins and hormones. In 1982, Butler *et al.* isolated two pregnancy specific proteins (PSPA and PSPB) from bovine placenta. Later PSPA was identified as α-fetoprotein, PSPB was found placenta-specific (Sasser *et al.*, 1986). In 1991, Zoli *et al.* purified and characterized a pregnancy-associated glycoprotein (PAG) from bovine fetal cotyledons later named PAG I_{67}. This PAG consists of four isoelectric variants (PI: 4.4, 4.6, 5.2 and 5.4) with a molecular mass of 67 kDa. Glycoproteins immunologically close to PAG I_{67} and PSPB were isolated from ovine placenta and later called oPAG1 (Zoli *et al.*, 1995) and oPSPB (Willard *et al.*). In 1998, Garbayo *et al.* isolated three PAGs (molecular weight: 55, 59 and 62 kDa) from mid pregnant goat placenta. Parallel to the protein purification, investigations were realized using molecular biology. After the isolation of the cDNA coding for PAG I67 and oPAG 1 it became clear that the pregnancy-associated glycoproteins are belonging to the aspartic proteinase family, sharing great sequence identity with pepsinogenes, cathepsin D, E, renin (5, 8, 13, 14). The PAG molecules purified until now are enzymatically inactive, because of mutations around the active site (Xie *et al.*, 1991). Later studies identified new DNA sequences encoding for PAG molecules in pig (Szafranska *et al.*, 1995), horse (Green *et al.*, 1994), zebra and in cat placenta (Gan *et al.*, 1997). As the PAGs are synthesized in mono, binucleate or syncitial cells of the trophectoderm (Xie *et al.*, 1991; Zoli *et al.*, 1992) and some of them are secreted on the maternal blood circulation, they can be good indicators of pregnancy and feto-placental well-being (Bohn, 1991; Sciarra *et al.*, 1963). Antisera produced in rabbit against bPSPB and PAG I_{67} allowed the development of radioimmunoassays for pregnancy detection in the bovine species from day 28 or 30 after

fertilization (Humblot *et al.*,1988; Zoli *et al.*, 1992). Also, the objective of this study was to compare the ability of three different antisera was compared to measure pregnancy-associated glycoprotein concentrations in plasma samples.

Blood samples were collected from the jugular vein into heparinized vacutainer tubes at 30–120 days after fertilization for pregnancy diagnosis (Laboratory Genes Diffusion, Douai, France). Plasma was removed after centrifugation (1000 g, 20 min) and stored at -20°C until assayed for PAGs. PAG I_{67} purified from bovine placenta was radiolabelled by the lactoperoxidase method with ^{125}I. The antisera used for the different RIA systems are shown in **table 1**. In the three RIA systems PAG I_{67} was used as standard (0.2-25 ng/ml).

The PAG measurements were realized according to the method of Zoli *et al.* (1992). Briefly, after the addition of appropriate dilutions of antisera, plasma samples and the standards were incubated overnight at room temperature. The following day, tracer (25000 cpm/tube) was added to all tubes, and they were incubated for 4 hours at room temperature. The separation of the free and bound fractions was done by centrifugation (1500 g, 15 min, 4°C) after the addition of sheep anti-rabbit-immunoglobulin, coupled to activated cellulose (DASP system). The supernatant was discarded, and the radioactivity in the pellet was determined using an LKB Wallac 1261 Multi Gamma counter. The regression and the variance analysis were realized using the PSI – Plot v. 4.0 and the GLM procedure of SAS, respectively.

Table 1. Antisera used in RIA1, RIA2 and RIA3.

Antiserum	Contains antibodies against	Dilution in RIA	System
497	PAG I_{67}	1:200000	RIA1
706	$PAG_{55,62}$	1:75000	RIA3
708	$PAG_{55,59}$	1:75000	RIA2

RIA 2 and RIA 3 systems gave significantly higher values than RIA 1 system. Moreover RIA 3 system gave higher values than RIA 2 system. Testing the same samples in RIA 1, RIA 2 and RIA 3 systems the regression parameters were calculated between RIA 2 and RIA 1, RIA 3 and RIA 1 and RIA 3 and RIA 2 systems. The correlation coefficient (r.) between the RIA 3 and RIA 1 systems (0.9403) was higher than between RIA 2 and RIA1 systems (0.9294), however the correlation between RIA 3 and RIA 2 was the highest (0.9738).

Our results suggest that antisera raised against $PAG_{55, 59, 62}$ purified from goat placenta recognize the bovine PAG molecules in maternal blood. The circulating forms of PAGs in cows are probably closer to PAG_{62} than to the other forms ($PAG_{55, 59}$). Presently we are trying to purify and characterize new forms of PAG from bovine placenta in order to improve the accuracy of early pregnancy diagnoses in cow.

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