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Changes in expression of the prolactin and growth hormone gene during different reproductive stages in the pituitary gland of turkeys

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Summary — The changes in levels of prolactin (PRL) and growth hormone (GH) in plasma and the pituitary gland and their transcripts were measured in turkey hens at different physiological stages by radioimmunoassay and dot blot hybridization analysis, respectively. The levels of tPRL mRNA in the pituitary gland increased from those of the immature group to the egg-laying group, reaching a maximum during the incubation and a minimum during the moulting stages. Changes in pituitary levels of PRL and PRL mRNA followed a similar trend and consequently were highly correlated ($r^2 = 0.83$), whereas a significant but lower correlation was observed between circulating and pituitary levels ($r^2 = 0.62$). Less significant changes were measured for tGH mRNA, with maximum and minimum levels measured in the pituitaries of egg-laying and non-laying hens, respectively. These data suggest that although changes in concentration of PRL are correlated with the reproductive stage of the turkey hen, coordinate changes in levels of GH are not.

turkey hen / prolactin / GH / mRNA / physiological stages

Résumé — Variations des taux d'expression des gènes de la prolactine et de la GH au niveau hypophysaire chez la dinde à différents états physiologiques. Les variations des taux d'ARNm dans l'hypophyse et des concentrations plasmatiques et hypophysaires de la prolactine et de la GH ont été quantifiées chez la dinde à différents états physiologiques, par la technique d'hybridation par *dot-blot* et par dosages radio-immunologiques. Le taux d'ARNm

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de la prolactine s'accroît avec acquisition de la maturité sexuelle et est respectivement maximal lorsque la dinde est couveuse et minimal chez les dindes chez lesquelles une mue forcée a été induite. Les taux d'ARNm de la prolactine et sa concentration hypophysaire évoluent parallèlement et sont par conséquent très fortement corrélés ($r^2 = 0,83$). De surcroît, bien que plus faible, une corrélation significative est également observée entre les concentrations plasmatiques et hypophysaires de prolactine ($r^2 = 0,62$). Globalement, les variations des taux d'ARNm de la GH sont de faibles amplitudes, avec des taux minimaux mesurés chez les dindes en arrêt de ponte et maximaux chez les dindes pondeuses. Aucune corrélation ($r^2 \leq 0,01$) n'est mise en évidence entre les taux d'ARNm de la GH et les concentrations hypophysaires et plasmatiques. Ces données montrent clairement que des variations de concentrations en prolactine sont associées à l'état physiologique et comportemental et au processus de reproduction chez la dinde alors que ce n'est pas le cas pour la GH.

dinde / prolactine / GH / ARNm / états physiologiques

INTRODUCTION

In the domestic turkey, elevated circulating levels of prolactin (PRL) have been associated with the onset and/or maintenance of incubation behaviour; however, the exact role(s) of PRL in this behaviour are not clear. The incubation phase of reproductive behaviour in the turkey is characterized by increased PRL levels, lower gonadotropin and ovarian steroid levels, ovarian regression, nesting activity, aggressive nest protection and anorexia (Burke and Dennison, 1980; Proudman and Opel, 1980; Zadworny et al, 1985). It is established that vasoactive intestinal peptide (VIP) is a major PRL-releasing factor in avian species (El Halawani et al, 1990; El Halawani and Rozenboim, 1993). Furthermore, immunoneutralization of VIP reduces circulating prolactin levels and results in the termination of incubation behaviour in chickens (Sharp et al, 1989) and turkey hens (El Halawani et al, 1995). The transition from the laying to incubating stage in bantam hens is accompanied by a ten-fold increase in the concentration of PRL mRNA (Shimada et al, 1991; Talbot et al, 1991). In addition, the enhanced release of pituitary PRL in incubating bantam hens is associated with increased proportions of PRL-secreting cells that acquire an enhanced secretory capacity (Lopez et al, 1996). The rise of circulating levels of PRL during incubation appears, therefore, to result from increased synthesis and release of pituitary PRL (Saeki and Tanabe, 1955; Cherms et al, 1962; Burke and Papkoff, 1980; Hoshino and Wakita, 1989); however, the exact mechanisms responsible for this rise are not known.

Growth hormone (GH), the other pituitary member of the PRL family, appears to be important for normal growth and development in avian species (reviewed by Scanes, 1984; 1987). Plasma levels of GH are high in young birds and low in the adult (Scanes and Lauterio, 1984). Higher levels of circulating GH have been measured in laying hens compared to incubating hens (Bedrak et al, 1981) and in young chickens which are fasted (Scanes, 1987). Conversely, it has been shown that both the pituitary content and synthetic rate of GH is significantly increased in incubating hens compared to laying hens. In addition, Kansaku et al (1994) noted that levels of GH mRNA also significantly increase in incubating chickens. Why pituitary content, but not release of GH during incubation, increases is not clear. However, turkey hens experience a profound anorexia and lose 20–30% of their body weight during incubation behaviour (Zadworny et al, 1985) and increased pituitary levels of GH may be related to the mobilization of body reserves during the rigours of the fast and/or to preparation for the termination of the fast. In support

of the latter, the transition from incubating to brooding behaviour results in a large, but transient, increase in circulating levels of GH (Wentworth et al, 1983) at the same time that normal ingestive behaviour resumes after the incubation of eggs (Zadworny et al, 1985).

The purpose of the present study was to further examine the changes in pituitary and blood levels of GH and PRL during the reproductive cycle of turkey hens in association with their expression in the pituitary gland.

MATERIALS AND METHODS

Animals

In the present study medium white (Betina strain) turkeys *(Meleagris gallopavo)* were assigned to the following physiological groups (n = 6 per group): 1) sexually immature hens (16 weeks old) kept under a photoperiod of 12 h light:12 h dark; 2) actively laying hens which were at the beginning of their second period of lay and had already laid eggs for 4 to 6 weeks; 3) hens which were at the end of their laying period and had laid eggs for over 20 weeks; 4) incubating hens that spent more than 70% of their time nesting and had not laid an egg for more than 3 weeks; 5) hens that stopped laying eggs after more than 20 weeks of production and had been out-of-lay for more than 3 weeks without any visible signs of moulting; and 6) hens which were force-moulted using a lighting schedule of 6 h light:18 h dark. With the exception of the sexually immature group (group 1), the hens were 90 to 98 weeks of age. All hens, except the immature and moulting groups, were maintained under a photoperiod of 14 h light:10 h dark. Blood samples (5 mL) were collected by brachial venipuncture into heparinized tubes and plasma was separated by centrifugation (2 000 x g, 10 min, 4 °C) and stored at –20 °C until analysed. The hens were killed by decapitation and the pituitary glands were removed, immediately snap-frozen in liquid nitrogen and stored at –70 °C until assayed for content of PRL and GH and their respective mRNAs.

Extraction of total pituitary RNA

All glassware was baked. Water for preparing solutions, used in RNA extraction and analyses, was treated with 0.1% diethyl pyrocarbonate (DEPC) and autoclaved for at least 3 h. All other instruments were either sterilized or treated with 1 M NaOH, 1% SDS solution to remove ribonucleases.

Individual frozen pituitaries were homogenized on ice in 15 mL Falcon tubes in ice cold homogenization buffer (220 µL of 10 mM Tris-HCl, 1 mM EDTA and 0.5% [vol/vol] Nonidet P-40, pH 7.5) (White and Bancroft, 1982) using a Brinkman polytron (Westbury, NY, USA) at high speed for 10–20 sec. Twenty µL of the homogenate were diluted with an equal volume of 10 mM Tris-HCl (pH 8.0) containing 0.1 mM PMSF, 1 µM leupeptin and 1 µM pepstatin and were stored at –70 °C for determination of PRL and GH content. To the remaining 200 µL of pituitary homogenate, 1 mL of denaturing solution was added according to Chomczynski and Sacchi (1987) and homogenized further on ice, with three bursts of 20 sec each. After adding 100 µL of chloroform and vortexing, the suspension was incubated on ice for 10–15 min. The phases were separated by centrifugation at 10 000 x g at 4 °C for 10 min, the aqueous layer was removed and RNA was precipitated by the addition of an equal volume of isopropanol. RNA was recovered by centrifugation (13 000 x g for 10 min at 4 °C). The resulting pellet was washed twice with 70% ethanol, dried and redissolved in DEPC-treated water. An equal volume of 8 M LiCl was added to the RNA samples followed by incubation at 4 °C for 16 h. RNA was recovered by centrifugation as before. The resulting RNA pellet was washed twice with 70% ethanol, dried and redissolved in DEPC-treated H_2O. RNA samples were adjusted to a final concentration of 6 mM $MgCl_2$, 80 mM Tris-HCl (pH 8.0), 10 mM NaCl and 2 mM DTT and then treated for 30 min at 37 °C with five units of RNAse-free DNAse in the presence of eight units of RNAguard (Pharmacia-LKB). After a phenol/chloroform step, the RNA was precipitated as before and dissolved in H_2O. Total RNA was

quantitated by A_{260} absorbance and the A_{260}/A_{280} ratio (> 1.9) was used as criterion for the purity of the RNA. Contamination of samples with DNA was verified by treating random RNA samples with RNAse and subsequent hybridization.

Northern blot analysis

In order to ascertain the quality of the extracted total RNA and estimate the size of the hybridizing bands, approximately 5 µg of pituitary RNA from turkeys of each physiological group were heat denatured at 60 °C for 15 min and size fractionated by electrophoresis in 1.5% agarose containing 2.2 M formaldehyde, 40 mM MOPS (3[N-morphol-inopropane-sulfonic acid]) at pH 7.0, 10 mM sodium acetate and 1 mM EDTA at 40 V overnight. The gels were stained with acridine orange. RNA was transferred to Gene Screen (NEN Research Products, Boston, MA, USA) by capillary action using 20 X SSC (1 X SSC: 0.15 M NaCl, 0.01 M sodium citrate) (Sambrook et al, 1989). The positions of the 28S and 18S on the filter were visualized using an ultraviolet (UV) lamp. RNA was cross-linked on the membrane by UV light irradiation for 5 min, followed by baking at 80 °C for 2 h.

RNA dot blot analysis

The relative concentrations of mRNAs for PRL and GH in the turkey pituitary were measured by the dot blot hybridization procedure described by White and Bancroft (1982). RNA samples were denatured by heating for 15 min at 65 °C in 10 X SSC containing 6% formaldehyde. For analysis, each RNA sample was diluted in a 96-well microtiter plate to yield a final volume of 100 µL. Four different amounts of total RNA (0.125, 0.25, 0.5 and 1 µg) were applied in duplicate, on Hybond-N nylon membranes (Amersham) using a 96-well manifold filtration vacuum apparatus (Schleider and Schuell, Keene, NH, USA). These amounts were chosen because preliminary experiments indicated a linear relationship between the RNA amounts blotted and the autoradiographic signal obtained after hybridization with radiolabelled tPRL cDNA probe. The filters were exposed to UV light for cross-linking to occur and baked as indicated earlier.

Northern blot and dot blot hybridization

The filters were prehybridized at 42 °C for at least 4 h and then incubated overnight with heat denatured, ^{32}P-oligolabelled cDNA probe at 5–10 ng/mL (final concentration < 1.5 x 10^6 cpm/mL). Both prehybridization and hybridization buffers contained 50% deionized formamide, 10% dextran sulphate, 5 X SSC, 0.1% SDS, 1 X Denhardt's solution (0.2% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone) and 100 µg/mL sonicated denatured salmon sperm DNA. Probes were prepared according to Feinberg and Vogelstein (1983) using an oligolabelling kit (Pharmacia-LKB). The filters were washed sequentially in: i) 2 X SSC, 0.1% SDS at 42 °C; ii) 0.5 x SSC, 0.1% SDS at 42 °C; iii) 0.1 x SSC, 0.1% SDS at 65 °C. Autoradiography was performed using Kodak XAR film (Eastman Kodak Co, Rochester, NY, USA) and Dupont Cronex Lighting Plus intensifying screens (Dupont, Wilmington, DE, USA) at –70 °C for various times in order to obtain a signal proportional to the amount of RNA applied for all the samples. For rehybridization analysis, the filters were boiled in 0.1 x SSC, 1% SDS for 30 min and hybridized as before. Care was taken that probes with approximately the same specific activities were used for every hybridization.

The filters were sequentially probed with rat 18S ribosomal RNA, tPRL (Karatzas et al, 1990) and tGH (Karatzas, 1993) cDNAs, respectively. Specific mRNAs were quantitated by densitometric scanning of the autoradiographs using a BioImager (MilliGen/Biosearch, Millipore, Canada). Each absorbance value was plotted as

a correlation of the blotted RNA and the slope was calculated by linear regression analysis. Values within the linear range were normalized according to the corresponding value of 18S (rat 18S cDNA). In order to account for differences between the hybridizations, strips of Hybond-N membrane were spotted with three concentrations of tPRL and tGH cDNAs (10, 30 and 60 pg each) and included in every hybridization. Values were then adjusted to the same baseline according to the control values and evaluated as stated earlier. As a negative control, turkey liver RNA was also included in every hybridization set. PRL and GH mRNA measurements are expressed as a ratio of PRL/18S or GH/18S and reported as arbitrary absorbance units.

Radioimmunoassays

Prolactin and GH in plasma and pituitary extracts were assayed in triplicate samples according to Guémené et al (1994) and Proudman (1984), respectively. All samples were assayed in a single radioimmunoassay and the intra-assay coefficients of variation were 4.2 and 3.1%, respectively.

Statistical analysis

Endocrinological data were analysed using an ANOVA procedure and Fisher PLSD (protected least significant difference) post hoc test if appropriate ($P \leq 0.05$, ANOVA). The standard deviation was used to express data variability. Analyses were performed using the StatviewTM II program (Abacus Concept Inc, Berkeley, CA, USA) for the Apple Macintosh computer.

RESULTS

Analysis of PRL and GH and mRNA in the turkey pituitary

The mRNA levels of PRL and GH in the turkey pituitary were measured by using the cDNA clones for tPRL and tGH, respectively. The integrity of the isolated RNA and estimate of the molecular size of the corresponding mRNAs are shown in figure 1. RNA extracted from individual pituitaries of sexually immature, laying, incubating and moulting hens was probed with 18S, tPRL and tGH cDNA probes in sequence. The PRL probe hybridized mainly to a single mRNA with an estimated molecular weight of about 1 000 bp in agreement with the size of the tPRL mRNA (Wong et al, 1992a). Two additional bands, which presumably represent unspliced transcripts (fig 1B, lane 3), were observed in the RNA extracted from the pituitary of an incubating hen. Rehybridization of the blot using tGH cDNA as a probe revealed that there was no cross-hybridization between tPRL and tGH. The tPRL and tGH mRNAs differed in size by about 100–200 bp relative to 18S and 28S RNA band. No tGH mRNA band was detected in the sexually immature hen even after longer exposure time (fig 1A, lane 1). This observation was not reproducible since the same sample gave a strong hybridizable signal when analysed by dot blot hybridization (fig 2).

PRL and GH gene expression in relation to the reproductive stage of the hen

The relative levels of PRL and GH mRNAs in pituitaries of hens at various physiological stages were measured by dot blot analysis; figure 2 is a representative blot of such an analysis. Since the same amount of total RNA was applied on the membrane, the density of each signal reflects the relative concentration of specific mRNA present in the turkey pituitary gland at the time of tissue collection; a summary of the analysis is presented in figure 3. The levels of tPRL mRNA in the turkey pituitary increased an average nine- and 13-fold, relative to sexually immature hens, during egg-laying (onset) and incubation (fig 3). During the late stages of egg-laying, levels of PRL mRNA decreased but were still significantly higher than in out-of-lay or moult-

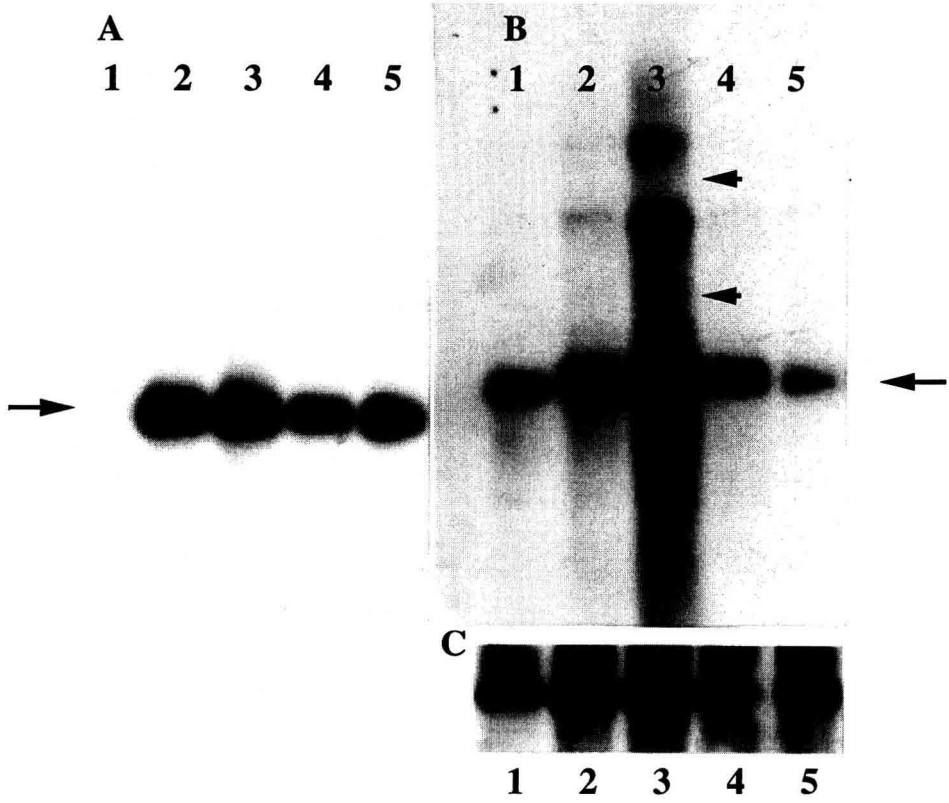

Fig 1. Northern blot analysis of total RNA (5 μg) isolated from individual pituitaries of immature (1), laying (2), incubating (3), out-of-lay (4) and moulting hens (5). The RNA was separated and, after transfer onto a membrane, was hybridized with ^{32}P-labelled tPRL cDNA **(A)**. The positions of the 18S and 28S are indicated on the right by arrowheads. The same filter was stripped of the probe and rehybridized with a tGH **(B)** or a rat 18S probe **(C)**. Autoradiography was for 20 h.

ing hens. In contrast, pituitary levels of tGH mRNA did not vary so markedly during the life of the turkey hen. Laying hens had significantly higher levels of GH mRNA than all the other stages with the exception of moulting hens.

Plasma and pituitary PRL and GH levels during the reproductive cycle of the turkey

Circulating levels of PRL were seven- and 16-fold higher in turkeys in the laying and incubation phase, respectively, than in the sexually immature hens (fig 3). There were no significant differences in plasma levels of PRL in the other groups. The levels of pituitary PRL showed the same relative trend as those of the plasma, being highest during the incubation phase and lowest in hens at the moulting phase. However, the fold increases were not similar. For example, incubating hens showed a four- and ten-fold increase in the pituitary gland and plasma, respectively, over the values of the sexually immature group. The transition from the incubation to

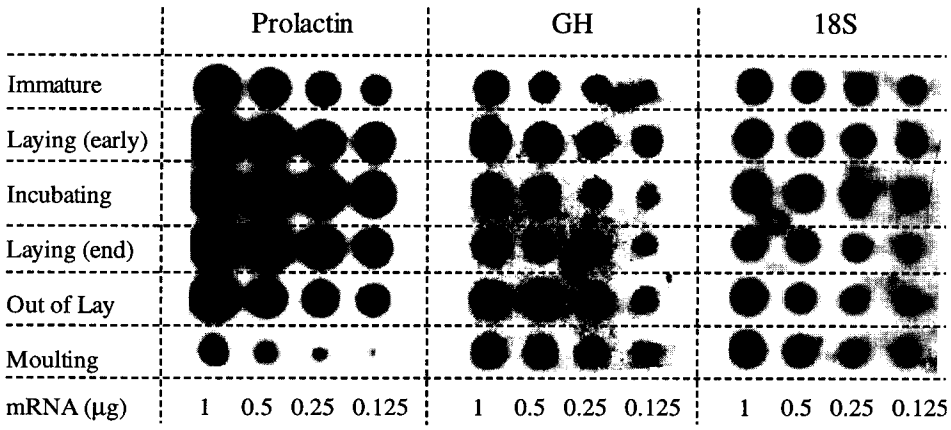

Fig 2. Levels of prolactin and growth hormone (GH) mRNAs in turkey pituitaries as quantitated by the dot blot hybridization technique. Total RNA spotted was sequentially hybridized with tPRL, tGH and 18S cDNAs. RNA samples were extracted from pituitaries of immature (1), laying (early: laying since 4 to 6 weeks) (2), incubating (3), laying (end-of-lay: laying since over 20 weeks) (4), out-of-lay (5) and moulting (6) hens.

the out-of-lay stage was accompanied with a 13- and four-fold decrease in plasma and pituitary levels of PRL, respectively.

Circulating levels of GH were not different throughout the physiological stages examined except for those measured in the plasma of immature hens. Although no significant changes were observed ($P > 0.05$), the levels of GH measured in the pituitaries of hens appeared to rise from a low level in the immature hens to an intermediate level in the egg-laying group and reached the highest levels in hens that were at the end of egg-laying.

Overall, tPRL mRNA levels were highly correlated with the levels of tPRL in the pituitary gland ($r^2 = 0.83$, $P < 0.05$), whereas, lower but still significant correlations were observed between tPRL mRNA and circulating levels or pituitary and circulating levels of PRL ($r^2 = 0.67$ and 0.62, respectively). No correlation was observed between GH mRNA, pituitary and plasma GH concentrations ($r^2 < 0.01$). An inverse correlation was observed in incubating hens which contained the highest PRL mRNA and the lowest GH mRNA levels.

DISCUSSION

The present study demonstrates that plasma and pituitary levels of PRL and PRL mRNA are markedly affected by the reproductive status of the turkey hen, wheras those of the related hormone, GH, do not vary to so great an extent. Thus, GH and PRL are not regulated coordinately during reproductive states in the domestic turkey.

Elevated concentrations of GH are associated with reduced caloric intake, essential fatty acid deficiency and low protein diets (Scanes, 1987), and short-term fasting results in increased GH secretion in young chickens (Scanes, 1987) and turkeys (Proudman and Opel, 1981). Since incubation behaviour is associated with aphagia and a 20–30% decrease in body weight (Zadworny et al, 1985, 1988), it has been proposed by Hoshino and Wakita (1989) that GH may be involved in lipolytic metabolism during incubation. These authors have shown that the synthetic rate of GH increases significantly in incubating over laying hens along with a concomitant small increase in pituitary content of GH. The

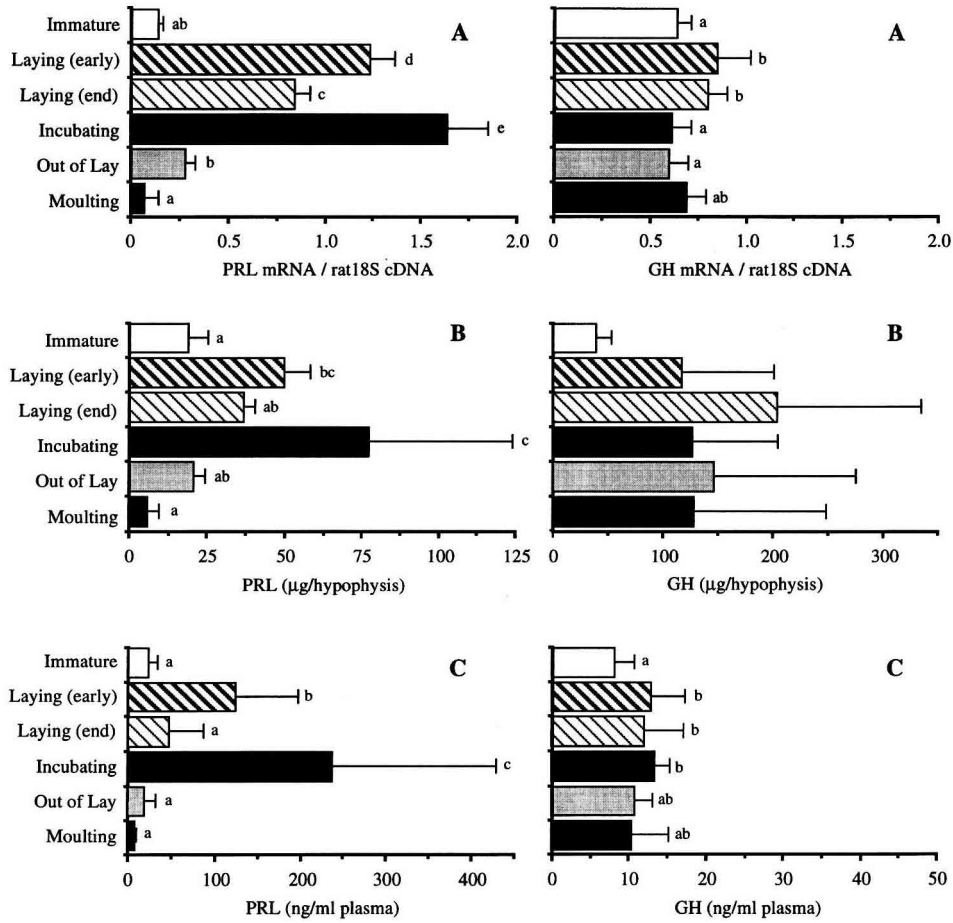

Fig 3. mRNA (A), pituitary **(B)** and plasma **(C)** levels of PRL and GH in turkey hens at different physiological stages were quantified. For mRNA analysis the autoradiograms (see fig 2) were scanned and individual values (PRL or growth hormone [GH]) were standardized to the levels of 18S and are reported as arbitrary absorbance units. Data are expressed as the mean ± SEM from four to five pituitaries of birds within the same physiological group. ^{abc} means with common superscripts are not statistically different from one another ($P > 0.05$).

results of the present study indicate that GH may not be directly implicated in the metabolic changes taking place during the incubation phase since circulating or pituitary levels of GH or GH mRNA do not change during this stage (fig 3). Similar results have been reported in the chicken (Kansaku et al, 1994). Therefore, factors other than, or in addition to, GH may be involved in the lipolytic process during incubation behaviour. However, we cannot exclude higher rates of GH turnover both within the hypophysis and the blood which might be associated with incubation behaviour. In support of the latter, Hoshino and Wakita (1989) observed a greater than three- fold increase in the synthetic rate of GH associated with incubation behaviour, whereas the rate of release was suppressed.

In addition, although blood levels of GH are lower during incubation than in egg-laying, a large increase in GH occurs at the termination of incubation behaviour (Wentworth et al, 1983). Thus, it is still possible that GH is associated with metabolic adaptation to the incubatory fast. Further studies are required to verify this hypothesis.

Although plasma and hypophyseal levels of GH and GH mRNA were not correlated in the turkey (fig 3, $r^2 < 0.01$) or the chicken (Kansaku et al, 1994), levels of PRL mRNA, hypophyseal and circulating immunoreactive PRL were correlated to the reproductive state of the hen (fig 3). Plasma and pituitary levels of PRL and its mRNA all increased from the low levels measured in the immature hens to reach a maximum during the incubation phase of reproductive behaviour and subsequently declined to reach a nadir in moulting hens. This is in agreement with results reported in the chicken (Shimada et al, 1991; Kansaku et al, 1994) and emphasizes the importance of PRL in reproductive behaviour and physiology in galliformes. Levels of tPRL mRNA were highly correlated with PRL pituitary content ($r^2 = 0.83$), which suggests that transcription and translation are closely coupled. Hoshino and Wakita (1989) reported that the transition from laying to incubating states in chickens was associated with about a two-fold increase in rates of PRL synthesis, hypophyseal content and rate of release. In the turkey, levels of mRNA, pituitary content and blood content increased 1.3-, 1.5- and 1.9-fold, respectively, which is in close agreement with Hoshino and Wakita's results. However, Talbot et al (1991) observed that plasma levels of PRL in the laying and incubating chicken hen were correlated with levels of pituitary PRL mRNA but not with pituitary content of PRL. Furthermore, Wong et al (1991, 1992a) reported that PRL mRNA abundance did not parallel the changes in either plasma or pituitary PRL in turkey hens at various stages of their reproductive cycle.

Experimental conditions, species, breed or assay differences may account for these discrepancies. In addition, it is notable that there are three major isoforms of PRL in the turkey (Corcoran and Proudman, 1991), and that the ratio of these isoforms changes in concert with the reproductive status of the hen (Bédécarrats et al, 1995). During incubation, the glycosylated isoforms are dominant and glycosylation is known to reduce immunoreactivity of mammalian PRL (Sinha, 1994). It is possible that the lower correlation observed between hypophyseal PRL mRNA and circulating levels of PRL ($r^2 = 0.67$) may be associated with an underestimation of immunoreactive PRL as has been observed with other PRLs (Pellegrini et al, 1988). This is underscored by the observation of Hoshino and Wakita (1989) using a disk electrophoretic method that synthesis and release of PRL are coordinately linked.

Enhanced mRNA stability, processing, transport of transcripts and high progressive transcription are all possible means of regulation (Jahn et al, 1984; Haisenleder et al, 1989) that affect the high levels of PRL mRNA levels measured in the turkey pituitary during the incubation phase (fig 3). Transcriptional regulators of avian PRL have not been studied in detail. In mammals the transcription factor Pit-1/GHF-1 regulates both PRL and GH genes (Ingraham et al, 1990). Wong et al (1992b) recently cloned the turkey counterpart of the mammalian Pit-1/GHF-1. They found no significant changes in the levels of Pit-1 mRNA during the reproductive life of the hen, including the incubating stage. Therefore, the increases in the PRL mRNA levels during the hyperprolactinemic phase in the turkey hen may be accounted for by the cooperation of additional transcriptional factors, but not Pit-1 alone, in mediating basal and hormonal-stimulated PRL gene transcription (Iverson et al, 1990; Wong et al, 1992b). For example, it has been shown that VIP regulates PRL mRNA levels in addition to acting as a physiological releasing factor

in the bantam (Talbot et al, 1991) and turkey hens (El Halawani et al, 1990b). The recent cloning of the turkey PRL gene (Kurima et al, 1995), including about 2 kb of its upstream regulatory sequence, should prove useful for studying transcriptional regulation.

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