Plasma Melatonin Levels in Relation to the Light-Dark Cycle and Parental Background in Domestic Pigs

By H. Andersson

Department of Clinical Chemistry, Centre of Reproductive Biology in Uppsala, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Introduction

The circadian rhythm of pineal melatonin, with an increased secretion during the night and low concentrations during the day, is mediating photoperiodic information to the neuroendocrine reproductive system in many non-tropical seasonal breeding mammals (Bartness & Goldman 1989).

The domestic pig breeds continuously, although seasonal variations in reproduction, with reduced fertility during late summer and autumn, have been demonstrated from many parts of the world (Claus & Weiler 1985, Love et al. 1993, and Peltoniemi et al. 1999). The period of seasonal infertility coincides with the anestrous period of the European wild boar (Sus scrofa) (Mauget 1982). Seasonal change in photoperiod has been suggested as an important factor causing these fertility problems, and artificial photoperiod has been shown to influence the timing of puberty in both gilts (Paterson & Pearce 1990) and boars (Andersson et al. 1998).
In the domestic pig, the reports of the existence of a typical circadian rhythm of peripheral melatonin have been contradictory, with only few studies reporting melatonin profiles that consistently change according to the light-dark phases (Paterson et al. 1992a, Andersson et al. 2000). Originally, no melatonin rhythm was found under short or long photoperiods (Reiter et al. 1987, McConnell & Ellendorff 1987, Minton et al. 1989), but day-night differences could be demonstrated in at least some animals in an equatorial photoperiod (McConnell & Ellendorff 1987, Minton & Cash 1990). Thereafter, several discrepant studies have been published (e.g. Diekman et al. 1992, Green et al. 1996 and 1999, Diekman & Green 1997, Bollinger et al. 1997, Bubenik et al. 2000), and the deviations of the results have been explained by variations of light intensity (Griffith & Minton 1992), by the great pig-to-pig variability (Green et al. 1996, Bollinger et al. 1997) and by inadequate assay methods (Klupiec et al. 1997, Andersson et al. 2000).

The amplitude of the nocturnal melatonin secretion in pigs appears to be lower than in most studied mammalian species (Andersson et al. 2000). If only a minor increase in melatonin secretion during the dark-phase is sufficient for a photoperiodic response on the reproductive system is not known.

The aim of this study was to investigate if parental background influence porcine melatonin in the light environment of a pig stable, and if sampling by jugular venipuncture can be used for evaluating individual melatonin profiles.

**Materials and methods**

**Animals and photoperiod**

Female Yorkshire pigs, 15 gilts from 5 litters and 16 sows, and 3 Hampshire boars were bled during winter (November-February) at 60°N (6-9 h of light). In August at 60°N (15-16 h of light), 48 crossbred (YxH) piglets, 24 females and 24 males (10-14 weeks of age), offspring of four gilts, 4 sows and 2 boars from the winter bleeding, were bled. The animals were kept in standard stable management with windows and additional light (light bulbs) during working hours (8:00-16:00). Daytime light intensity varied depending on weather conditions between 150-300 lux, with occasional higher intensities. Overall nighttime light conditions were very low for the gilts and piglets (<5 lux). The sows and boars had low-intensity night illumination (light bulbs) creating a nighttime light intensity between 5-10 lux.

**Plasma sampling**

Three daytime samples and 3 nighttime samples were collected by jugular venipuncture into heparinised tubes between 10:00-15:00 and 22:00-03:00, respectively, from each animal, with approximately hourly intervals. Nighttime light intensity varied somewhat depending on lunar phase and weather conditions, such as cloudiness and snow. To facilitate sampling during the night, dim red light and a small flashlight were used. Thus, it is not possible to exactly say which light intensity the animals were exposed to at each moment of sampling, although any direct light exposure of the pigs’ eyes was avoided at all times. After collection the samples were centrifuged and stored at -20°C until analysed for melatonin content.

**Melatonin assay**

Plasma melatonin was analysed by radio immunoassay (Bühlmann Laboratories AG, Schönenbuch, Switzerland). Before assay, 1 ml portions of controls and samples were extracted twice in 4.5 ml of diethyl ether. The tubes were then shaken for 1 min and put into a freezing bath. The supernatant was decanted and the solvent removed by evaporation to dryness in a 37°C water bath, whereupon the residue was
dissolved in 1 ml of incubation buffer. Duplicate aliquots (400 µl) of standards, extracted controls and extracted plasma samples were pipetted into the tubes, followed by 100 µl of anti-melatonin antiserum (Kennaway G280; caprine against melatonin conjugated to bovine thyroglobulin, see Vaughan, 1993), and 100 µl of the 125I-melatonin tracer. The tubes were then incubated for 20 h (± 4 h) at 2-8°C. While stirring the second anti-body, 100 µl of the suspension was added to the tubes, after which they were incubated at 2-8°C. After 15 min 1 ml of cold, distilled water was added to the tubes, which were then centrifuged at 2-8°C. After 15 min the supernatant was removed and the radioactivity of the tubes was counted in a gamma counter for 2 min. Serial dilutions of pig plasma containing high concentrations of melatonin produced displacement curves parallel to the standard curve. The intra-assay and inter-assay coefficients of variations for 20 assays, were 13.1% and 8.2% (2.4 pg/ml), and 8.4% and 8.0% (19.5 pg/ml), respectively, and the sensitivity of the assay was 0.3 pg/ml (intercept of maximal binding - 2 S.D.). Using reversed-phase column extraction, the manufacturer calculated the minimal detectable concentration to be 0.3 pg/ml. The specificity of the assay has been evaluated by Bhlmann Laboratories AG and all measured compounds show less than 0.05% cross-reactivity. Selected samples were reanalysed on a later occasion, in order to ensure assay repeatability.

Statistics
Statistical analyses were performed by analysis of variance by MIXED procedures (SAS Institute Inc. 1997) and least square means option was used to compare different means. Melatonin levels from the winter bleeding were tested for variance of time-of-day (day versus night), sampling order within time-of-day, sex and age within sex with individual animal as random effect. The melatonin concentrations of the gilts from the winter bleeding were furthermore analysed in a model with time-of-day, sampling order within time-of-day and mother (litter) as fixed effects (effect of fathers could not be considered as it partly overlapped with litter) and individual animal as random effect. Melatonin levels from the summer bleeding were initially analysed in a model with time-of-day, sampling order within time-of-day, sex, father, mother (litter) within father as fixed effects and individual animal within father as random effect. As no significant variation was associated with sampling order within time-of-day, sex or father, melatonin from the piglets were reanalysed in a model with time-of-day, mother(litter) and the interaction between mother(litter) and time-of-day as fixed effects and individual animal within father as random effect. Melatonin concentrations from both sampling occasions were analysed for the effects of time-of-day, sampling order within time-of-day, sex and age within sex as fixed effects and individual animal as random effect.

Results
Nighttime melatonin concentrations were higher than daytime melatonin concentrations (Table 1), whereas no effect of sampling order could be discerned at either bleeding occasion. The adults and the young animals were bled at different times of the year. When wild and domestic pigs were compared in 4 seasons, the melatonin rhythm was entrained by the photoperiod of the season whereas no effect of sea-

Table 1. Daytime (10:00-15:00) and nighttime (22:00-03:00) plasma melatonin concentrations (least square means ± s.e.m.). (N=82)

|       | Day   | Night | P-value |
|-------|-------|-------|---------|
| Melatonin (pg/ml) | 2.7 ± 0.8 | 14.4 ± 0.8 | p<0.001 |
son on melatonin levels could be found (Tast et al. 2001). There was no difference in melatonin levels between adult and young animals in this study.

**Adults**

The 3 adult Hampshire boars had higher nighttime (23.5 ± 2.9 pg/ml; least square mean ± s.e.m.) and daytime (9.8 ± 2.9 pg/ml) melatonin concentrations than the 31 Yorkshire sows and gilts (night: 14.1 ± 0.9 pg/ml, day: 3.3 ± 0.9 pg/ml) (p<0.05). There was no clear difference between gilts and sows in melatonin levels. In spite of the low numbers of animals per litter, the gilts from one of the litters had higher plasma melatonin concentrations than the gilts in 3 other litters (Table 2).

**Piglets**

Among the 48 piglets, the effect of father was not quite significant (p=0.12) and there was no difference in melatonin concentrations between the male and female piglets. There was an interaction between time-of-day and litter (mother) (p<0.01) as nighttime but not daytime plasma melatonin concentrations differed between litters (Fig. 1).

**Discussion**

In a pig stable environment, domestic pigs showed a nocturnal increase in plasma melatonin secretion. Nighttime plasma melatonin levels differed between litters, which indicates that the great individual variations in the amplitude of nocturnal melatonin secretion, observed in this species (e.g. Andersson et al. 2000, Tast et al. 2001) has a genetic background. Jugular venipuncture, which is a commonly used bleeding method in pigs, requires restraining of the animal. The stress that is associated with being restrained leads to increase of heart rate, catecholamine, cortisol and β-endorphin levels etc. (Roozen et al. 1995). Some of these stress reaction, such as plasma cortisol concentrations, can be expected to have been increasing during the bleeding period, yet no differences in plasma melatonin level between first, second and last time of sampling could be discerned, indicating that the stress and handling as such during the bleeding did not disturb the melatonin measurements. As all animals showed a higher average nighttime melatonin concentration than daytime level, and there was a high individual variation in nighttime melatonin levels, this indicates that plasma samples collected by jugular venipuncture can serve as a basis for evaluating melatonin profiles from a large number of animals. However, occasional high melatonin concentrations were observed during the day. Since plasma sampled by indwelling jugular catheters revealed only low to undetectable daytime melatonin concentrations, using the same assay (Andersson et al. 2000, Tast et al. 2001), the random higher measurements in this study possibly were caused by a cross reaction with some factor(s), which may

### Table 2. Daytime (10:00-15:00) and nighttime (22:00-03:00) plasma melatonin concentrations (least square means ± s.e.m.) in gilts from different litters.

| Litter (n) | Day (pg/ml) | Night (pg/ml) |
|------------|-------------|---------------|
| Litter 1 (n=4) | 2.6a ± 2.7 | 23.2a ± 2.7 |
| Litter 2 (n=2) | 1.9a ± 3.8 | 8.7b ± 3.8 |
| Litter 3 (n=4) | 0.6a ± 2.7 | 6.4b ± 2.7 |
| Litter 4 (n=3) | 0.9a ± 3.1 | 10.3b ± 0.9 |
| Litter 5 (n=2) | 2.5a ± 3.8 | 15.0ab ± 3.8 |

Values within a row with no superscript in common differ significantly (p<0.05)
have entered the blood sample as the needle passes through the epidermis and subcutaneous layers at the time of venipuncture. Irrespective of cause, this emphasises the importance to use multiple sampling in order to correctly evaluate the individual melatonin profiles, when jugular venipuncture is applied.

The 3 adult Hampshire boars in this study showed higher plasma melatonin concentrations than the adult females, although a clear nighttime increase in melatonin secretion was observed in both sexes. Daytime melatonin concentrations consistently elevated above the detection limit were only observed for the 3 adult boars (not among the male piglets). Although higher pineal concentrations of melatonin have been observed in male compared to female Siberian (also called Djungarian) hamsters (Phodopus sungorus; Niklowitz et al. 1996), interpretation of results from so few animals must be made with caution, especially since the gender in this case overlapped with the breed. Extra-pineal melatonin is synthesised in e.g. the gastrointestinal tract, but its contribution to circulating melatonin levels is controversial (Heuther 1993). Though the melatonin levels of the boars over all were significantly higher than the plasma concentrations of the adult females, there was no sex difference in the extent of the night-time melatonin increase. Therefore, the possible sex differences in melatonin concentrations probably have no importance for the role of melatonin as an endocrine signal of darkness. However, increased diurnal levels of the main urinary melatonin metabolite (6-sulfatoxymelatonin) have been observed among Siberian/Djungarian hamsters that are reproductively unresponsive to photoperiod (Niehaus & Lerchl 1998).

Although the melatonin rhythm in sheep is highly repeatable within the individual (Chemineau et al. 1996), the amplitude of nocturnal melatonin shows high inter-individual variability (Malpaux et al. 1987), which is caused by a genetic variability in the synthesis of pineal melatonin (Zarazaga et al. 1998a and Zarazaga et al. 1998b). In contrast to an earlier study (Andersson et al. 2000), there was no significant effect of fathers in this study. Therefore, it can only be suggested that inter-individual variability in night-time melatonin concentrations reflects a genetic variation. Differences in nighttime melatonin seemed to be depending on the

Figure 1. Daytime (10:00-15:00; white horizontal bars) and nighttime (22:00-03:00; dark horizontal bars) plasma melatonin concentrations (mean ± sem) in piglets from different litters (n=48, six piglets per litter, 3 males and 3 females). The mothers’ melatonin levels are marked with open circles (A-D are sows and I-IV are gilts).
sibling group among the gilts, although the number of gilts per sibling group was low (2-4 animals per litter). But, since the same influence of litter was seen among the piglets (6 animals per litter), the variation in amplitude of night-time melatonin secretion between sibling-groups could be confirmed. The offspring used in this study had spent their short lives in an almost identical environment. Furthermore, the older piglets were no longer kept together with their litter mates at the time of the bleeding, but were mixed with piglets from other litters according to sex. Thus, the social group did not overlap with the sibling group among these piglets. Age and weight of the piglets overlapped with litter, as a result of the study design.

In lambs a melatonin pattern that reflects the light-dark cycle is present already at 3 weeks of age and the amplitude of nighttime melatonin secretion increases between 6 and 27 weeks of age (Claypool et al. 1989). In contrast, in female rhesus monkeys the nighttime amplitude of melatonin secretion decreases during pubertal development (Wilson & Gordon 1988). Among the piglets, however, there was no clear trend of an increase or decrease of the amplitude of night-time melatonin concentrations with age, as both the highest and the lowest average night-time melatonin concentrations were found among the older (and heavier) piglets. Together, this supports the hypothesis that the differences in melatonin pattern between litters observed in this study, probably is a result of the genetically determined capacity for pineal melatonin synthesis which has been described in sheep (Zarazaga et al. 1998a).

As seasonal infertility is a management problem for the pig producers, it was important to see whether a night-time increase in melatonin secretion was observed in a conventional pig stable environment. This study showed increased melatonin secretion during the dark hours as is the case in other animals (Reiter 1993). The nocturnal increase in pigs is relatively low compare to many other studied species, but the average nighttime melatonin concentration was always higher than the average day-time concentration for each individual animal. Studies on the effects of photoperiod or exogenous melatonin administration on pig reproduction have shown varied results (e.g. Krealing et al. 1983, Lee et al. 1987 and Paterson et al. 1992b). Whether the low nocturnal secretion of melatonin observed among some sibling groups influences the response to photoperiod or melatonin is not possible to state, since no reproductive parameters were measured in this study. However, a circadian rhythm in melatonin, with a clear elevation during the dark phase, is required for transferring photoperiodic information in all seasonal breeding mammals (Reiter 1993).

In conclusion, this study demonstrates that domestic pigs of different ages, breeds and sex show a night-time elevation of melatonin secretion in a pig stable environment. Although always higher than the daytime base levels, the increase in melatonin secretion during the night is small in some animals. Furthermore, the amplitude of the nighttime melatonin secretion differed between litters, which suggests a genetic background.

Acknowledgement

The author wish to thank the Department of Animal Breeding and Genetics, SLU for the use of their breeding herd, Eva Norling, Ulf Hermansson and Carola Jansson for help with the collection of blood samples and all the rest of the staff at Funbo-Lövsta for taking such good care of the animals, Karin Burvall is thanked for all the hard work with the melatonin assay.

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Sammanfattning
Melatoninivär i plasma hos tamsvin i relation till ljus-mörker och härkomst.

För att studera melatoninutsöndring hos grisar i stallmiljö, samlades 3 dagsprover (10.00-15.00) och 3 nattprover (22.00-03.00) plasma med hjälp av venpunktion från 15 gyltor, 16 suggor, 3 galtar och 48 kultingar (24 honor och 24 hanar från 8 kullar) och analyserades på melatonininhåll. Melatonininkoncentrationerna under natten var högre än under dagen (p<0,001), men ingen effekt av provtagningsordning kunde ses. De 3 galtarna hade högre melatoninivärer än de 31 gyltorna och suggorna, både under dag och natt, medan det inte fanns någon skillnad mellan gyltor och suggor. Fyra gyltor från samma kull hade högre melatoninivärer under natten än gyltorna från 3 andra kullar (p<0.05). Bland de 48 kultingarna var det skillnad mellan kullarna i melatoninivär under natten (p<0,01), medan effekten av fäder inte var riktigt signifikant (p=0,12). Det fanns ingen skillnad i dagsnivärer mellan kullarna och ingen skillnad mellan hanar och honor. Sammantaget visar denna studie att grisar i stallmiljö har en ökad melatoninutsöndring under natten. Hos somliga djur var amplituden i melatoninutsöndring under natten lika medan skillnad mellan kullarna, vilket tyder på en genetisk variation.

(Received September 5, 2000, accepted January 31, 2001).

Reprints may be obtained from: Department of Clinical Chemistry, PO Box 7038, S-750 07 Uppsala, Sweden. E-mail: Hakan.Andersson@klke.slu.se, tel.: +46-18-671614, fax: +46-18-309565.
Pseent address: MCR Human Reproductive Sciences Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh, EH3 9ET, UK. E-mail: h.andersson@hrsv.mrc.ac.uk, tel: +44 (01) 131 229 2575, fax: +44 (01) 131 228 5571.

Acta vet. scand. vol. 42 no. 2, 2001