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Active Immunization Against cVIP and It's Role on the Pattern of Sequence Length and Pause Days in Domestic Hen (*Gallous domesticus*)

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Abstract: Chicken vasoactive intestinal peptide (cVIP) is a hypothalamic PRL (PRL) releasing factor in birds and it plays an important role avian reproduction. Our objective was to determine whether active immunization against cVIP would increase the sequence length, decrease intersequence pause days, and there by improving egg production in White Leghorn (WLH) by decreasing the prolactin (PRL) concentration during the active period of productive cycle in birds. WLH birds at 13 weeks of age were divided into two experimental groups consisting of 21 birds in control and immunized birds. At 17th week of age, 21 birds were immunized with synthetic chicken VIP (conjugated with keyhole limpet haemocyanin KLH-cVIP), followed by three booster doses at four weekly intervals with a total of four immunizations. Controls were given placebo in place of immunogen. cVIP antibody titers were consistent till the age of 72 weeks in immunized birds. Active immunization of the birds against cVIP significantly ($P<0.01$) decreased the circulating PRL during and after the withdrawal of immunizations and PRL levels were found to decrease up to the age of 72 weeks of age, resulting in significantly lower incidence of shorter sequences, with significantly ($P<0.01$) lower number of intersequence pause days, egg laying sequence lengths in birds immunized against cVIP. Total number of pause days were significantly reduced in immunized birds compared to controls. Egg production increased by 7.00% in the immunized birds over the control birds with the available resources under normal husbandry practices. It is observed that the laying pauses are mainly due to fluctuations in the concentration of PRL above physiological levels in circulation and keeping the PRL hormone under check will influence egg production, pause days, sequence length, persistency in egg production with almost an egg a day. It is conclude that the physiological pauses that occur during ovulatory sequences can be disrupted effectively using active immunization against cVIP, which modulates PRL levels, that may interfere with the follicular recruitment and subsequent oviposition thereby improving egg production in white leghorn hens.

Key words: Active immunization against cVIP, sequence length, inter sequence pauses, PRL, White leghorn

Introduction

A high rate (intensity) and persistency of egg laying are associated with a low incidence of broodiness. This is the term used to describe persistent nesting or incubation behavior, which is a part of a complex of maternal behaviors. The expression of incubation behaviour is thought to be a consequence of sequential interactions between estrogen, progesterone and prolactin (PRL) acting on the brood patch and hypothalamus. Brood patch formation is initiated by an increase in circulating estrogen, progesterone originating from maturing ovarian follicles at the onset of lay (Sharp *et al*., 1989). These steroids also act on the hypothalamus to induce the nesting behavior that is associated with the laying of each egg. Tactile stimuli from the eggs to the brood patch are transmitted by neural pathways to the hypothalamus to stimulate the activity of vasoactive intestinal polypeptide (cVIP) neurons that stimulate PRL release from the anterior pituitary gland. Increased plasma PRL acts on the brood patch to complete its formation, on the ovary to inhibit steroidogesis, and on the hypothalamus, to prolong nesting behaviour converting it to incubation behaviour. Once incubation behaviour is established, the ovary regresses and concentrations of estrogen and progesterone in blood decrease. Incubation behavior is maintained by increased plasma PRL acting through PRL receptor located in the hypothalamus (Sharp *et al.,* 1988). Several experiments were conducted to disrupt the broodiness in birds through active immunization against PRL /cVIP in birds. The gene responsible for broodiness has been isolated and at present the broodiness is almost eliminated from the commercial flocks having White Leghorn birds. However, till now, no systematic study was conducted to unravel the basic physiological mechanism (as explained in case of broodiness) involved in taking pauses taking between the sequences of egg lay in domestic hen. In this study, our main objective was to lower the PRL levels, by immunizing the birds against cVIP, and examining the egg laying characteristics such as, age at first oviposition, sequence length and intersequence pause lengths in hens. Further, recent advances in understanding the physiology of broodiness and

persistency of egg laying provided an insight to, to unravel the basic physiological mechanism involved in taking pauses between the sequences of egg lay, pattern of oviposition, and inter sequence pauses through active immunization against cVIP. Even if we can reduce the pause days by 1 or 2%, there is a tremendous increase in egg production in commercial poultry farms with the available resources under normal husbandry practices.

Materials and Methods

Experimental birds: The study was conducted with 42 White Leghorn birds of strain were housed in individual cages (1'x 1' x 1') from 13 to 72 weeks of age under two-tier battery system. The birds were kept under constant light (2 lux at the level of the eye) to eliminate all diurnal variations from the pattern of oviposition. All hens were fed on the same layer ration (16 per cent CP and 11.72 MJ ME Kg^{-1}) as per the standard NRC (1994) recommendations and water was made available throughout the day.

Immunization: Chicken VIP was conjugated by the gluteraldehyde method to Key hole limpet haemocyanin (KLH ; Sigma , USA) as described by Lerner *et al.* (1981). The first dose of the immunogen containing 125µg cVIP was given as KLH-cVIP conjugate in 1 ml of Freund's adjuvant made up to 2 ml with distilled water. The mixture was emulsified and intradermally injected (@ 2 ml/bird) into the lateral thoracic wall under the wings. Subsequent boosters were given with KLH –cVIP conjugate containing 25µg cVIP in Freund's incomplete adjuvant. Control hens were injected placebo in place of immunogen. Treatment was repeated at four weeks interval with a total of four immunizations (@ 2 ml/bird) spanning between 17th to 32nd weeks of age.

Sampling of blood: The birds were bled on weekly intervals from 13th to 72nd week of age as well as immediately before and 15 days after each immunization and brachial venous blood (~2 cc) was collected from each bird in heparinised tubes. The blood was centrifuged at 2500 rpm for 15 minutes and plasma was harvested and stored at –20^{o}C until assayed for PRL and tested for the presence of antibodies for cVIP.

Egg production records: Egg production was recorded for each hen at the same time each day for a continuous 378 days period. Egg sequence length and the number of egg sequences were determined from oviposition records following the procedure described by Blake and Ringer (1987). The number of eggs laid on successive days by a particular hen determined the length of each sequence and the number of pauses in each hen's oviposition determined the number of sequences. For each hen the length of laying sequence was determined on the day the last egg of the current clutch was laid. If a hen did not experience a pause during that period no value was recorded or else the actual number of pauses observed during that period was recorded.

Radioimmunoassay of PRL: The Chicken PRL hormone and antisera used in the assay were provided generously by Dr. Parlow (NIADDK, USA). The hormone PRL was iodinated following the procedure of Sharp *et al.* (1989). The assay was carried out following the detailed procedure of Koprowski and Tucker. (1971). The antiserum was used at a final dilution of 1:4,00,000. PRL standards ranged between 50 to 1000 ng/ml. The bound and free fractions were separated using anti rabbit γ-globulin raised in goat at a final dilution of 1:100. The Intra and inter assay coefficients of variation for PRL were 7.22% and 9.50%, respectively.

VIP-Binding study: cVIP binding study was carried out as per the method described by Mauro *et al.* (1992). Antibody titers were checked periodically using 125 I-Monoiodinated cVIP. The titres were checked periodically using 125- Monoiodinated cVIP till 72 weeks of age in immunized birds, which gave more than 60% of the binding.

Statistical analysis: All quantitative data were subjected to one-way analysis of variance and correlation using Microsoft statistical package. Statistical significance was set at $P< 0.01$. Group data are presented as Mean ± S.E.

Results

Effect of active immunization against VIP on PRL concentration: The immune response, measured by the percentage binding of monoiodinated cVIP to plasma at a dilution of 1:1000, with the maximum binding of 15.3±1.86% after the administration of final booster dose at 29th week of age. The plasma samples collected from control birds contained no detectable antibody to cVIP. PRL concentration in control and immunized birds were estimated using RIA. The concentration of PRL from 13th week of age was increased in both the groups (Fig. 1). Following active immunization against cVIP at 17th week of age, profiles of PRL significantly decreased during and after the immunization till 72 weeks of age, where as PRL levels were higher in the control group throughout the 72-week period. During peak egg production (beginning from 28th weeks of age to 30th weeks) the circulatory levels of PRL were lower in both the groups.

Effect of modulation of PRL through active immunization against cVIP on egg production, sequence and intersequence pause: All the hens in the two groups started to lay eggs by 19th week of age. The mean age at first egg was 130.10±0.86 days and 134.85±0.10 days in the immunized and control group

Table 1: (Mean ± S.E.) Egg production, egg sequence and pause days between control and cVIP immunized birds from 19th to 72nd weeks of age in White Leghorn birds

	cVIP Immunized birds	Control birds
No. of birds	21	21
Age at first oviposition	130.10[a] ± 0.86	134.85[b] ± 0.10
No. of days	378[a]	378[a]
Number of laying days	321.90[a] ± 2.13	295.05[b] ± 2.46
Total number of sequences	35.72[a] ± 2.21	68.96[b]± 2.78
Maximum sequence length (days)	71.10[a] ± 2.74	23.35[b] ± 4.43
Mean sequence length (days)	8.4[a] ± 0.34	4.21[b] ± 0.49
Mean pause length	2.67[a] ± 0.18	3.95[b] ± 0.91
Total Pause days	56.11[b] ± 1.58	82.95[a] ± 1.29
Percentage of egg production	85.15[a] ± 1.13	78.15[b] ± 1.33
Difference in percentage of egg production	7.00	

[a,b] Means having at least one common superscript do not differ at 1% level (P<0.01). [NS] Non Significant.

respectively (Table 1). There was a significant decrease in the age at first egg between the immunized and control birds, with a significant increase (P<0.01) in the number of laying days in the immunized birds (321.90±2.13 days) as against control group (295.05± 2.46 days). The total number of sequences (35.72± 2.21) in immunized birds were significantly lower than the controls (68.96±2.78), with a maximum sequence length of 71.10±2.74 days in immunized birds (continuous laying without pauses) compared to the controls with 23.35±4.43. Mean sequence length of 8.40±0.34 to 4.21±0.49 days in immunized and control birds respectively. The total numbers of sequences were significantly higher in control group (82.95±1.29) compared to immunized birds (56.11±1.58). Mean inter sequence pause length (skipped days/days without an egg) over a period of 54 weeks was lower in immunized birds (2.67±0.18 days) as against controls (3.95±0.91 days). Significantly (P<0.01) higher percentage of egg production was observed in immunized birds 85.15±1.13% compared to controls 78.15±1.33%. There is a significant increase in egg production of about 7% in immunized birds over controls.

Discussion

The results of the present study show that active immunization of white Leghorn hens with KLH – cVIP was able to prevent the rise in circulating PRL during age at first egg up to peak egg production and increase the egg production potential of the birds till 72 weeks of age, compared to control hens. The effect of immunization against cVIP on decreasing circulatory concentration of PRL is consistent with the role of cVIP as a potent releaser of PRL in avian species, a concept supported by cVIP stimulation of PRL secretion and PRL mRNA abundance by turkey or chicken pituitary cells *in vitro* (Xu *et al.*, 1992) and the reduction in plasma PRL and PRL mRNA content after passive or active immunization with cVIP antibodies *in vitro* (Chen *et al.*, 1997). The PRL releasing activity of cVIP appears highly specific since it does not stimulate GH or LH release from the anterior pituitary gland acting at both transcriptional level and at the level of mRNA stability (Tong *et al.*, 1998) probably through specific membrane receptors present in the anterior pituitary gland.

Direct measurement of cVIP in the hypophysial portal blood in the turkey demonstrates that the amount present is directly related to concentration of PRL in the peripheral circulation (Youngren *et al.*, 1996). The highest levels of cVIP in the hypophysial portal blood and PRL in the peripheral circulation are seen in incubating hens and the lowest levels of both hormones are seen in sexually inactive hens. Immunomodulation of circulating PRL release in incubating bird's blocks photo induced PRL secretion and thus improves egg production in turkey hens and in 56 week old Taihe hens (Chen *et al.*, 1997).

In this study, active immunization against cVIP resulted in significant (p<0.01) reduction in PRL concentration in the peripheral circulation that sustained throughout the experimental period (Fig. 1), which supports the earlier findings of El-Halawani *et al.* (1995) in turkey hens that, when turkeys immunized with cVIP conjugated to keyhole limpet haemocyanin showed lower PRL levels compared to control birds.

Age at first egg: There was a significant decrease in the age at first egg between immunized and control birds. It is observed that external stimuli such as supplemental lighting (Robinson *et al.*, 2001) influence the age at first oviposition in addition to the genetic constitution of individual birds. The early age at first oviposition observed in the present study might be due to the synchronized effect of continuous lighting and immunization with KLH-cVIP. This is in conformity with the reports of El-Halawani *et al.* (1995) in turkey hens, where immunization with cVIP and photo stimulation resulted in increased egg production. Our observations

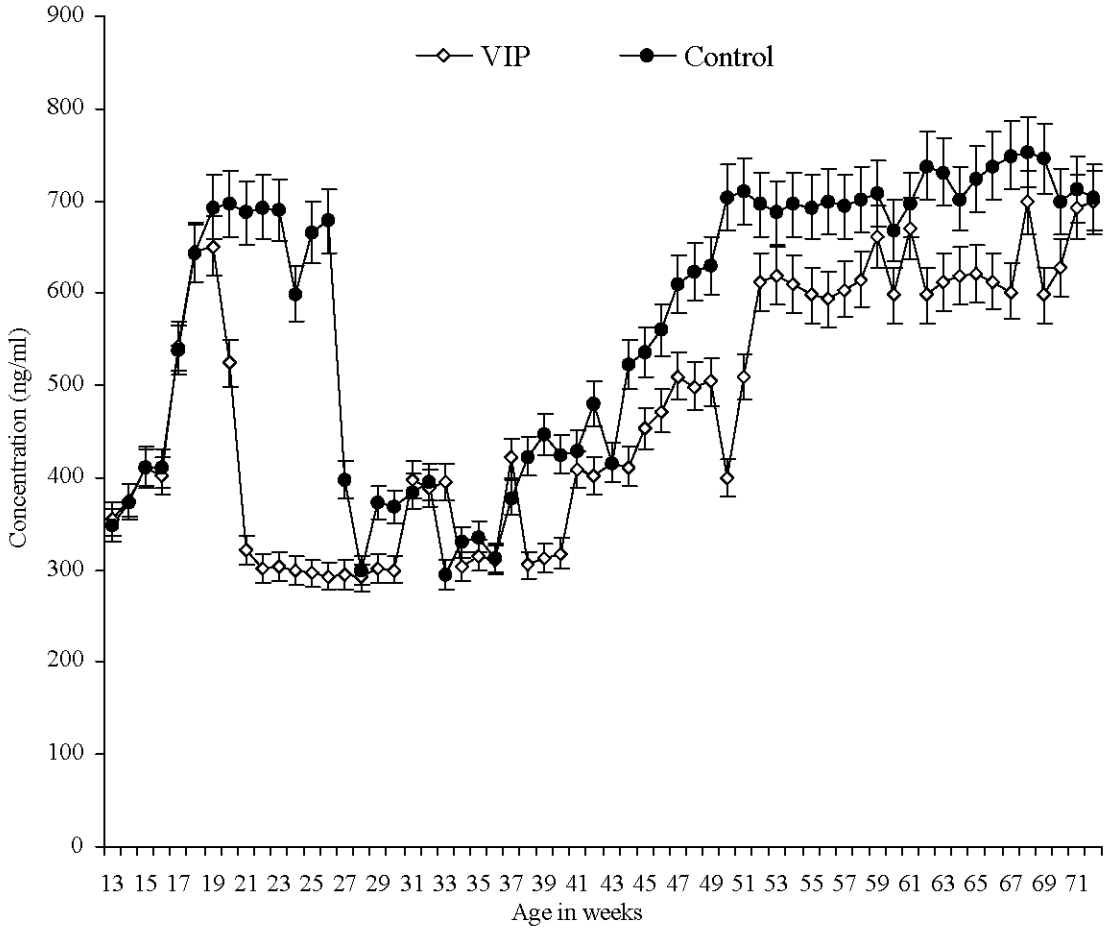

Fig. 1: Plasma PRL concentration (ng/ml) , showing the changes in the circulating levels in birds immunized against vasoactive intestinal peptide (n=21) compared to the control birds (n=21)

were contradicting with Robinson *et al.* (1993), where early age at first oviposition impairs egg production since greater allocation of energy is towards ovary in young age which may cause impairment in ovarian control as observed in broiler breeds (Bedecarrates *et al.*, 1997). And also the limited body reserves would have been depleted by the time of peak egg production (Robinson *et al.*, 1990). We conclude from our observations that high egg production was primarily a function of higher rates of lay throughout the laying period of 72 weeks rather than the age at sexual maturity.

Egg Sequence length: Immunization of birds against cVIP increased the number of laying days in immunized birds compared to the control birds with significantly fewer egg sequences (Table 1). Convincing evidence has been presented implicating increased PRL secretion as the cause of reduced circulating gonadotrophins, ovarian regression and the shift from egg laying to the incubation phase of reproductive cycle in the hen (Crisostome *et al.*, 1998). This is further fortified by the findings of Ogawa *et al.* (1977) that intravenous injection of mammalian PRL in hens 6-7 hours before the expected second ovulation, blocks the second ovulation but not when given 5 or 8 –14 hours before the second ovulation. In our study we have observed reduced laying pauses and longer sequences in immunized birds, which may be due to the low concentration of PRL, as higher levels of PRL have negative effect on gonadotrophic and gonadal hormones.

Increase in egg production is also due to the rate at which follicles enter their final phase of rapid growth, which is also under the influence of PRL. At high concentration, PRL interferes with follicular steroidogenesis in avian species (Dajee *et al.*, 1998) and only minimal amounts are required for normal growth. This fact is also emphasized in studies with human granulosa cells that failed to grow and secrete progesterone *in vitro* in the absence of PRL even in the presence of adequate amounts of gonadotrophins. In our earlier study we observed a negative correlation between PRL with progesterone and estradiol 17 β

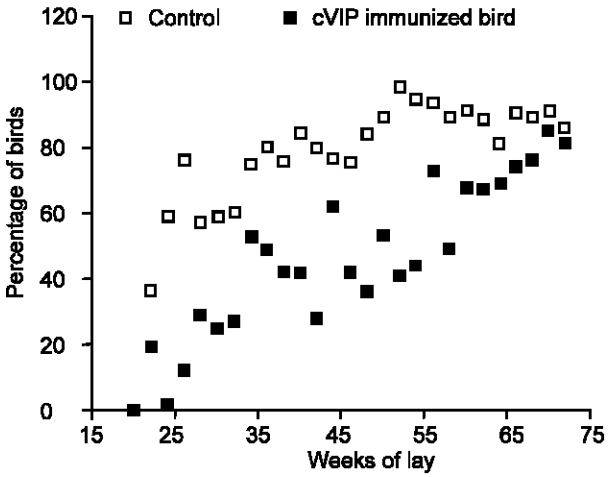

Fig. 2: Percentage of birds showing inter sequence pauses in control and cVIP immunized birds.

(Reddy *et al.*, 2002) which indicates that higher PRL levels have negative effect on steroid hormones, which are essential for egg yolk synthesis, albumen secretion and calcification of egg.

Lower concentration of these hormones delays egg formation and oviposition in birds. This may be the reason for shorter sequences of egg lay in controls.

Laying pauses: Increase in intersequence pause length of more than 2 days duration may be the consequence of reduced rate of follicular maturation and its subsequent recruitment into the hierarchy following ovulation which is partly regulated by FSH (Etches and Cheng, 1981). PRL at high levels suppresses the FSH induced estradiol production through the aromatase enzyme system (Wang *et al.*, 1980) resulting in reduced steroidogenic potential within the follicles. This reduced steroidogenic potential is not able to produce progesterone sufficient to elicit a positive feedback of LH required for ovulation (Dorrington and Gore-Langton, 1981). In our earlier studies we also observed an increase in the concentration of estradiol-17β and progesterone in plasma of birds treated with anti PRL agent (bromocriptine) compared to control birds (Reddy *et al.*, 2002). In support of our statement that modulation of PRL either by using bromocriptine (Reddy *et al.*, 2001) or by active immunization against cVIP / PRL in turkeys (El-Halawani *et al.*, 1990) overcomes the inhibitory effect of PRL on follicular development and subsequent oviposition with significantly lower number of pause days in immunized birds (Fig. 2), further, we observed that at necropsy that ovaries of immunized birds had greater number of yellow yolk follicles compared to the control group. This may explain the cause for longer sequences and reduced laying pauses in the treated birds. However, the occurrence of more than 10 days of laying pauses in birds of both groups may be due to the genetic constitution of individual birds. The mechanism responsible for ovulation and its failure, which lead to skipped days has been much studied but not clarified. Even though the role of PRL in occurrence of broodiness in turkey and bantam hens is well known it was not extended to laying chicken, particularly in relation to laying pauses in between clutches, which has been emphasized in this study. In the present study the immunization against cVIP during the initial weeks of laying was able to control egg production throughout one reproductive cycle up to 72 weeks of age in White Leghorn hens. This is supported by the observations of Guemene and Williams (1994) that low initial concentrations of PRL (far from exerting any deleterious effects on egg production) is closely associated with longer persistency of egg laying and that the hormonal profiles for a given hen during the first ten weeks of the laying cycle may provide productive information for future changes in the physiological status. We conclude that the physiological pauses that occur during ovulatory sequences can be disrupted effectively using active immunization against cVIP, which checks and keeps PRL concentration at physiological limits for eliminating the negative effects of PRL on hypothalamo-hypophysial-gonadal axis for higher follicular recruitment and subsequent oviposition thereby improving egg production and improve egg laying potential in White Leghorn hens.

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