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Received October 14, 1958. P.S.E.B.M., 1959, v101.

Pituitary Prolactin Levels in Laying, Incubating and Brooding Pheasants (*Phasianus colchicus*).* (24817)

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The ring-necked pheasant (*Phasianus colchicus*) in Wisconsin can be classified as an "indeterminate layer"(1). This classification is used for birds which lay until accumulation of eggs releases the incubation response. Pheasants in Wisconsin drop a considerable number of eggs prior to laying in clutch and incubating(2). The physiological control of initiation of incubation and broodiness has been studied. Riddle and Lahr(3) and Lehrman(4) indicated that progesterone induces incubation in ring doves. Only recently has progesterone been identified in birds(5,6,7). Association of prolactin with broodiness is well known(8,9,10). Crispens(11) was able to induce broodiness in hen pheasants prior to and during reproductive season by injecting prolactin. His criterion for broodiness was acceptance of chicks by a treated hen. One can postulate that initiation of incubation is controlled by prolactin. Actual relationships between pituitary prolactin levels during incubation and brooding is not well established. Understanding of various physiological changes during the reproductive period is necessary before activities of bird can be properly interpreted. We conducted this study to determine pituitary prolactin levels during various stages of incubation and brooding.

Materials and methods. Hen pheasants were autopsied in various stages of reproduction in 1955 and 1956. Anterior pituitary glands of some of these birds were removed, weighed and immediately frozen for prolactin bioassay. In 1955, 3 to 5 glands were chosen from birds in the following stages: laying; 8th, 16th and 20th days of incubation; and 3rd, 7th and 11th days after hatching. The same procedure was followed in 1956 when 3 to 5 glands were chosen from birds in both laying and non-laying condition 4th, 8th, 12th, 16th and 20th days of incubation; and 3rd, 7th and 11th days after hatching. In late July, immediately following breeding season, glands from birds in each stage of reproduction were pooled and homogenized in a glass homogenizer. The homogenate was diluted with distilled water to concentration of 20 mg fresh weight tissue/cc. These aqueous homogenates were injected intracutaneously over crop glands of 8-week-old white king pigeons, a modification of technic of Lyons and Page(12). With this method it is possible to test 2 homogenates in each bird, 1 for each crop gland. Prior to assay homogenates were coded. Crop glands to test each homogenate, were selected at random. Each preparation provided test material for at least 2 assay glands. Water placebo was injected over 4 crop glands to provide additional control. The spot over each crop gland around

* This study was financed in part by Wisconsin Conservation Dept. with Federal Aid to Wildlife Restoration funds under Pittman-Robertson Project and N.I.H.P.H.S. Data in this article were used in thesis presented to Univ. of Wisconsin Graduate School in partial fulfillment of requirements for Ph.D. degree.

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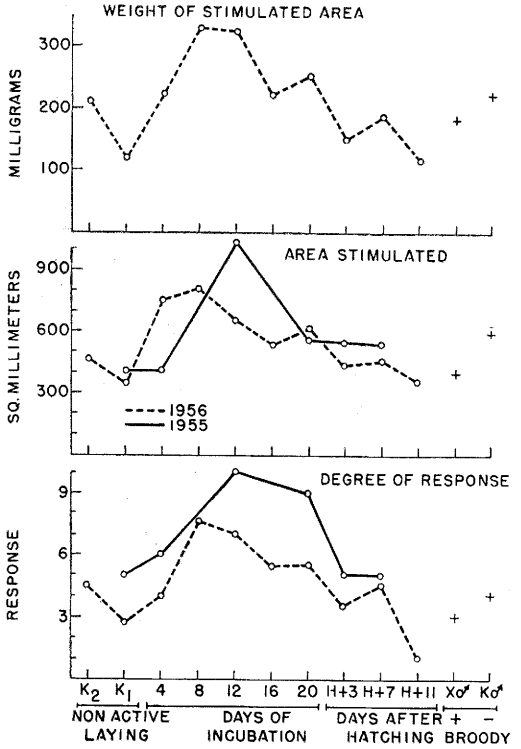

FIG. 1. Response of pigeon crop glands to pheasant pituitary homogenates from various stages of reproduction.

which injections were made was marked by injecting India ink. Injections of homogenates were made daily at noon for 4 days. Each crop gland received 0.125 cc/day and 4-day total of 10 mg of pituitary tissue. Each day's material was injected in a quartile clockwise around the marked site. Birds were autopsied on 5th day and gland response was determined in 3 different ways. First, degree of development was determined on 10 point basis. Absence of development was recorded as 0, initial development of rugae was rated 1 to 3. Additional thickening of stimulated area and production of pigeon milk were rated 4 to 10. Second, length and width of stimulated area of crop gland were measured by stretching the gland over base of small beaker and holding it up to the light. Sufficient tension was applied to keep tissue taut without tearing. Product of length and width of stimulated area is expressed as mm^2 of gland stimulated. Third, the stimulated area of crop gland was excised and weighed. Results were decoded following examination of crop glands at time of autopsy.

Results. Examination of Fig. 1 reveals that form of curves by all 3 criteria of crop response were essentially the same. Pituitary prolactin levels as measured by pigeon crop responses were lower for laying birds than for post reproductive non-laying controls. Prolactin content rises steadily during early incubation to a high level about 8th to 12th day. Responses from pituitaries of birds during late incubation and brooding, indicate a progressive decline from high mid-incubation level. The initial rise in pituitary prolactin during incubation can be interpreted as increased rate of formation by the pituitary. Broodiness in cock pheasants is a relatively rare occurrence. In 1956, however, we measured pituitary prolactin levels in 2 broody cocks as compared to 2 non-broody cocks. After 20 days incubation the pituitary prolactin levels in broody cocks were lower than those of non-broody cocks, as measured by our bioassay technic. These data suggest that pituitary prolactin levels do not continue to increase with advance of incubation, instead they tend to decline as incubation progresses.

Discussion. Godfrey and Jaap(13) demonstrated that broodiness in chickens can be terminated by estrogen. This could indicate that prolactin production or release is suppressed by high estrogen levels. The laying bird would normally be accepted as one in which estrogen titers were high. The commonly accepted mechanism in mammals, however, is one in which high estrogen levels stimulate production of prolactin. Our data indicate that a sudden acceleration in prolactin production occurs in response to internal or external stimuli. The stimulus necessary to elicit intense broodiness following this period of high prolactin production, may be considerably lower than in pre-incubation birds. The real expression of broodiness occurs in the latter half of incubation and after hatching, though individual differences exist between birds (Breitenbach, Meyer and Nagra, in manuscript). The action of prolactin on broodiness was first demonstrated by Riddle, Bates and Lahr(8). Many authors have since contributed information establishing correla-

tion between brooding behavior and prolactin stimulation. See Collias(14) for review and evaluation of the literature.

Relation of hormone production by bird pituitary to target organs and their secretions during reproduction is complex. Prolactin's inhibition of FSH production has been reported(15,16). The effects of progesterone in interrupting lay and initiating molt are counteracted by prolactin(17). Progesterone activity has been reported for extracts of avian blood(5,7) and in extracts of ovaries from laying fowl(6). Some workers reported effects of exogenous progesterone on bird physiology. Among them, Riddle and Lahr (3) showed that both progesterone and testosterone induce incubation and apparently stimulate release of prolactin by dove pituitary. Participation in incubation itself, stimulates prolactin secretion(18). Molt is characteristically inhibited during the brooding period (Breitenbach and Meyer, in manuscript). Inhibitory action of prolactin on molt process, reported by Juhn and Harris (17), is the probable explanation for delay in onset of molt. As brooding season progresses prolactin levels decline and the molt proceeds. Lehrman and Brody(19) demonstrated synergistic action of estrogen and progesterone on the dove oviduct and indicated that progesterone induces immediate incubation. Recently, however, in the turkey, it has been reported that progesterone seems to stimulate lay and interrupt broodiness(20). Obviously the relation of hormonal factors such as estrogen and progesterone to initiation of incubation is an area deserving more investigation.

Utilization of above information and our data tend to support the following hypothesis: Through accumulation of ovulated follicles in the ovary, increasing amounts of a substance (perhaps progesterone) are produced. This material causes production of prolactin by the pituitary. External stimulation is supplied by nest or accumulating eggs of clutch and is responsible for release and increased production of prolactin. The prolactin in turn inhibits FSH production and indirectly estrogen output. The influence of prolactin is expressed in broody behavior of the hen in relation to nest, and ultimately to the chicks.

Summary. Pituitary glands were removed from hen pheasants in various stages of reproduction—laying, non-laying, incubating and brooding and bioassayed for prolactin content. Prolactin levels rise rapidly during early incubation from levels observed in laying birds. Peak prolactin levels were noted from 8th to 12th days incubation. Prolactin content declined rapidly during latter portion of incubation and throughout the first 11 days following hatching. This occurred despite the fact that late incubation and post-hatching are periods of most intense brooding activity. Non-laying birds showed slightly higher pituitary prolactin levels than did laying birds. Pituitaries of 2 broody cocks were examined after 20 days incubation and showed less prolactin than pituitaries of 2 non-broody control cocks. It is suggested that early high prolactin levels condition the bird for subsequent brooding stimuli. The relationship to control mechanisms is discussed.

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Received October 30, 1958. P.S.E.B.M., 1959, v101.

Mammalian Cell Cultures for Study of Influenza Virus. I. Preparation of Monolayer Cultures with Collagenase.* (24818)

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Enzymatic treatment of cells, developed(1) for routine passage of cells in continuous culture, has been employed widely for primary dispersion of cells from tissue. While parts of some tissues, notably renal cortex, are dispersed readily by application of trypsin, other tissues are more or less refractory. Consequent restriction of cell types that can be dispersed efficiently by trypsin from tissue hampers *in vitro* study of some viruses, comparison of virus susceptibility of cells *in vivo* and in primary culture, and direct clonal derivation of cell lines from tissue. This paper describes the use of collagenase for preparation of primary monolayer cultures of lung, a tissue usually poorly dispersed by trypsin, for study of influenza viruses.

Materials and methods. Solutions included a) GKN (solution of glucose, potassium chloride and sodium chloride in concentrations appropriate to Hanks' solution)(2); b) Hanks' balanced salt solution brought to pH 7.4-7.6 with sodium bicarbonate; and c) nutrient medium containing 20% serum of the same species as the tissue cultivated in YEM (3) with penicillin and streptomycin added to final concentrations of 50 units and 100 μg per ml. *Tissues* were obtained as a) human and swine lungs from fetuses aged approximately 80 (14 cm) and 70 days respectively, and b) lung from an adult American Dutch rabbit about 250 days of age. Fetal swine lungs were obtained from miniature swine developed for research(4), and from sows randomly selected at an abattoir (by kind cooperation of Dr. Eldon G. Hill of the Hormel Institute, Univ. of Minnesota). Animals were opened aseptically and lung or other tissue removed to sterile Petri dishes containing about 2 ml of Hanks' solution. *Enzymes* in GKN were sterilized by passage through Selas #02 filters. Collagenase (Worthington Biochemical Corp., Freehold, N. J.) was prepared in stock concentration of 0.1% for use as 100 μg per ml (0.01%); trypsin as Difco Yeastolate was prepared in stock concentration of 20 mg per ml (2.0%) for use as 2 mg per ml (0.2%). *Preparation of dispersed cell suspensions. Lung* tissue was minced into 1-2 mm fragments cut by crossed scalpel blades handled like scissors; portions of bronchial tree separable from parenchyma were discarded. Fragments were rinsed repeatedly with Hanks' solution to remove erythrocytes, transferred to a screw-capped flask containing a Teflon-covered magnetic stirring bar, covered to an approximate depth of 30 mm with collagenase solution, and incubated for 10 min in a water bath at 37°C. Flask contents then were agitated by magnetic stirring for 2 hours in a 37°C incubator. An essential precautionary measure to prevent cell damage from heat was the insertion of an inverted Petri

* Aided by Grants from Frederica Beard Memorial Grant for Cancer Research, Am. Cancer Soc., Nat. Cancer Inst., N.I.H., and National Fn.

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‡ This work was done in partial fulfillment of requirements for Ph.D. degree in Bacteriology and Immunology, Univ. of Minnesota.