GENERAL AND COMPARATIVE ENDOCRINOLOGY 22, 54–61 (1974)

Effect of Hypothalamic Implantation of Puromycin on Photostimulated Testicular Growth in the Japanese Quail (*Coturnix coturnix japonica*)

MASARU WADA

Misaki Marine Biological Station, University of Tokyo, Misaki, Kanagawa-ken, 238-02, Japan

Received March 20, 1973

When a mixture of puromycin and cholesterol (1:50 by weight) was implanted in the anterior part of the nucleus tuberis, photostimulated testicular growth and development of the cloacal protrusion were impaired. When the implants were placed in the posterior part of the nucleus tuberis testicular growth was not affected, but development of the cloacal protrusion was inhibited. Implants in other hypothalamic areas had no effect on the development of the testes or the cloacal protrusion. These results suggest that the nucleus tuberis is the site of synthesis of the gonadotropin-releasing factor(s) and that their production and release are induced by photostimulation.

Although it is generally accepted that in mammals the hypothalamus produces releasing factors that control adenohypophysial functions, the hypothalamic nuclei which produce these factors have not been identified. This is also the case with the avian hypothalamohypophysial system. Lesion experiments designed to find center(s) controlling the adenohypophysis suggest that the ventral hypothalamus is involved in the regulation of gonadotropin secretion in birds (Wilson, 1967; Stetson, 1969a,b; 1972a,b; Sharp and Follett, 1969). However, lesions destroy not only neuronal perikarya but also their fibers, glial cells, ependymal cells and their processes. Thus, it is difficult to determine the exact sites producing releasing factors by this method.

Puromycin is known to inhibit protein synthesis *in vivo* and *in vitro*. For example, puromycin injected into the subarachnoidal space inhibited the formation of neurosecretory material in the rat (Zambrano and De Robertis, 1967). Recently, we have shown that puromycin implanted in the paraventricular nucleus and supraoptic nucleus of the rat inhibited the formation of neurohypophysial hormones in the neurosecretory neurons in these areas, resulting in increase of water intake and urine excretion (Saxena *et al.*, 1972).

In the present study, the author implanted puromycin into various parts of the quail hypothalamus and tried to find the nuclei which responded to puromycin implantation by inhibiting photostimulated testicular growth. The nucleus effected by the implantation may be assumed to be the site of production of the gonadotropin-releasing factor (GRF).

MATERIALS AND METHODS

Male Japanese quails (*Coturnix coturnix japonica*) were obtained from a commercial source at the age of 21 days and kept under a short daily photoperiod of 8L 16D (lights on from 800 to 1600) for about 2 weeks before puromycin implantation. Implantation was performed at the age of 32–38 days. On the day of implantation, the birds were transferred to a long

daily photoperiod of 16L 8D (lights on from 800 to 2400), and kept under this photoperiod for 10 days to stimulate testicular growth. Food and water were given ad libitum.

Puromycin dihydrochloride and cholesterol (Ch) (Merck and Co.) were mixed in a small amount of ether in ratios of 1:500 and 1:50 by weight. Ether was then evaporated and the mixture was tamped into the terminal portion of a 28-gauge stainless steel tube (inside diameter about 0.2 mm). The tubes containing the mixture were then implanted bilaterally into the hypothalamus approximately 0.3–0.5 mm lateral to the third ventricle. The tips of the tubes were placed in the desired positions with the aid of a stereotaxic apparatus and X-ray equipment. The tubes were fixed to the cranial bone by dental cement and two anchoring screws. Birds with tubes containing only Ch served as controls. The details of the implantation technique were described in a previous paper (Wada, 1972).

Body weight and cloacal protrusion were noted daily during photostimulation. It has already been established that development of the cloacal protrusion depends entirely on testicular androgen (Sachs, 1969). On the day of the start of the experiment, the feathers in the cloacal region were plucked to facilitate the cloacal measurements, as suggested by Sachs (1969). The cloacal protrusion was measured from the middle of the posterior lip of the cloaca to the posterior edge of the protrusion and across the protrusion at its widest point. The relative cloacal protrusion area (mm^2/100 g BW) was calculated from these values and the percent increase of the cloacal protrusion was calculated from the difference between initial (day of implantation) and final determinations of the relative areas. After 10 days' photostimulation, the birds were killed by decapitation, and the hypothalamic regions were dissected out after removal of the skull with its implanted tubes. The hypothalamic region was fixed in Bouin's solution, embedded in paraffin, and sectioned serially at 10 μm. The sections were stained doubly with paraldehyde fuchsin and toluidine blue O (Asai *et al.*, 1969). The testes, adrenals, thyroids, and adenohypophyses were weighed at autopsy. The testes were fixed in Bouin's solution, sectioned at 6 μm and stained with hematoxylin and eosin.

The sites of the tips of implanted tubes were determined from the histological sections and projected on a diagram of the midsagittal plane of the quail brain. The names of the hypothalamic nuclei were obtained by reference to the brain atlas of the domestic fowl by van Tienhoven and Juhász (1962).

RESULTS

Intact Group (Groups 1 and 2)

After 10 days' photostimulation, the testes and cloacal protrusions of all the intact photostimulated birds (Group 1) developed greatly, while those of the nonphotostimulated birds (Group 2) did not (Table 1). The seminiferous tubules of photostimulated birds became larger and contained spermatocytes. Those of the nonphotostimulated birds were very compact and contained only spermatogonia. The adrenals and thyroids showed no significant difference in weight between the two groups. The adenohypophyses of the photostimulated birds were heavier than those of the nonphotostimulated birds (Table 1).

Cholesterol Group (Group 3)

Cholesterol implants were placed in the basal hypothalamus, the nucleus tuberis, nucleus hypothalamicus posterior medialis, and nucleus hypothalamic inferior. The testes of the cholesterol-implanted quails developed markedly; they did not show a significant difference in weight from those in the photostimulated group. However, the cloacal protrusion was less developed than in the intact photostimulated group ($p < 0.01$) (Table 1). This may be attributed to the implant operation. The adrenals, thyroids, and adenohypophyses of this group were not significantly different in weight from those of the intact photostimulated quails.

Puromycin-Cholesterol (1:500) Implanted Group (Groups 4, 5 and 6)

Thirty-one quail were implanted with tubes containing a mixture of puromycin and cholesterol (1:500); these were implanted in the hypothalamus and other areas (Fig. 1). In all but one quail development of the testes and cloacal protrusions was similar to that in the cholesterol implanted birds (Table 1). In the exceptional quail development of the cloacal protrusion (60.0% increase) was delayed, although the development of the testes (256 mg) was about normal for the group. In

TABLE 1

EFFECT OF PUROMYCIN IMPLANTED IN THE HYPOTHALAMIC REGION ON PHOTOSTIMULATED TESTICULAR GROWTH AND THE WEIGHT OF OTHER ENDOCRINE ORGANS

Group	Number of birds	Sites of implantation	Body weight (g)	Organ weight (mg/100 g BW)				% Increase of cloacal protrusion
				Testes	Adrenals	Thyroids	Adenohypophysis	
1	(10)	Nonphotostimulated control	104 ± 2.1[b]	39.0 ± 12.5	7.75 ± 0.25	6.48 ± 0.40	0.94 ± 0.05	−7.0 ± 2.4
2	(10)	Photostimulated control	106 ± 1.5	497 ± 89.7	8.30 ± 0.29	7.01 ± 0.49	1.44 ± 0.26	284.5 ± 17.6
3	(16)	Cholesterol (Ch) control	98 ± 2.8	363 ± 30.0	7.94 ± 0.42	6.97 ± 0.61[e]	1.25 ± 0.07	190.8 ± 17.8
4	(8)	P-Ch (1:500)[a] in nucleus (N.) tuberis	99 ± 3.4	369 ± 36.3	7.60 ± 0.48	5.18 ± 1.79	1.39 ± 0.05	205.9 ± 23.5
5	(9)	P-Ch (1:500) in periphery of N. tuberis	100 ± 2.8	379 ± 32.1	6.67 ± 0.65	6.37 ± 1.00	1.31 ± 0.40	190.7 ± 25.6
6	(14)	P-Ch (1:500) in other areas	96 ± 2.4	411 ± 34.2	8.45 ± 0.44	6.14 ± 0.49[e]	1.36 ± 0.07	199.1 ± 15.5
7	(5)	P-Ch (1:50) in anterior N. tuberis	88 ± 4.4	209 ± 32.8[c]	10.85 ± 0.75[d]	4.76 ± 0.52	1.32 ± 0.06	38.2 ± 16.2[d]
8	(5)	P-Ch (1:50) in posterior N. tuberis	89 ± 1.9	261 ± 26.1	9.24 ± 0.48	7.71 ± 2.11	1.33 ± 0.07	93.9 ± 18.2[d]
9	(10)	P-Ch (1:50) in periphery of N. tuberis	90 ± 2.2	367 ± 45.3	9.26 ± 0.22[c]	7.27 ± 0.45	1.23 ± 0.06	156.2 ± 31.8
10	(24)	P-Ch (1:50) in other areas	92 ± 1.3[c]	379 ± 19.3	9.14 ± 0.42	6.44 ± 0.45	1.32 ± 0.05	177.0 ± 11.0

[a] Mixture of puromycin and cholesterol by weight.

[b] Mean ± standard error.

[c] Significant ($p < 0.05$) compared with Ch control group.

[d] Highly significant ($p < 0.01$) compared with Ch control group.

[e] In these groups, thyroid glands of one bird were not recovered.

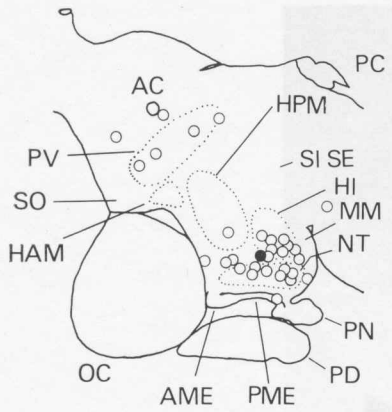


FIG. 1. The sites of puromycin and cholesterol (1:500) implants in the hypothalamic region 0.3–0.5 mm lateral to the third ventricle. The sites of the tips of the tubes and the nuclei are projected on the midsagittal plane. ○, An implant causing no effect on testicular growth and cloacal development; ●, an implant inducing a moderate effect on cloacal development. AC = anterior commissure; AME = anterior median eminence; HAM = nucleus hypothalamicus anterior medialis; HI = nucleus hypothalamic inferior; HPM = nucleus hypothalamicus posterior medialis; MM = nucleus mammillaris medialis; NT = nucleus tuberis; OC = optic chiasm; PC = posterior commissure; PD = pars distalis; PME = posterior median eminence; PN = pars nervosa; PV = nucleus paraventricularis magnocellularis; SI-SE = stratum cellulare internum and stratum cellulare externum region; SO = nucleus supraopticus.

this quail, the implant was placed in an anterior peripheral region of the nucleus tuberis (Fig. 1). The weights of other endocrine organs were the same as in the control birds.

Microscopic examination revealed that the neuronal perikarya in the regions around the tips of the implanted tubes containing puromycin of this concentration were apparently the same as those of the Ch group.

Puromycin-Cholesterol (1:50) Implanted Group (Groups 7, 8, 9 and 10)

Puromycin at a concentration of 1:50 was implanted into the hypothalamus of 44 quails.

In Group 7, five birds received implants in the anterior part of the nucleus tuberis. In nearly all these birds the development of testes and cloacal protrusion was delayed. The only exception was one quail whose testicular growth was normal but cloacal growth was disturbed (334 mg and 79.8% increase, Figs. 2a,b, and 3). Five birds of Group 8 received implants in the posterior part of the nucleus tuberis. Three of them showed excellent testicular growth but delayed development of the cloacal protrusion. The remaining two birds bearing implants in the ventral region of the posterior nucleus tuberis had delayed testicular growth; the development of the

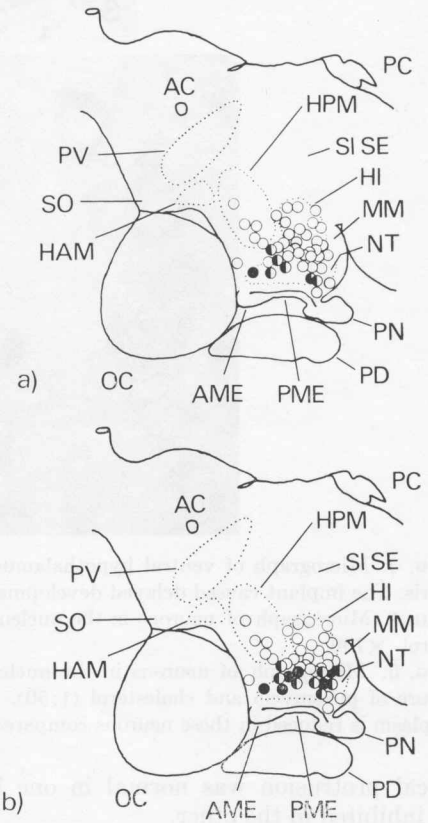
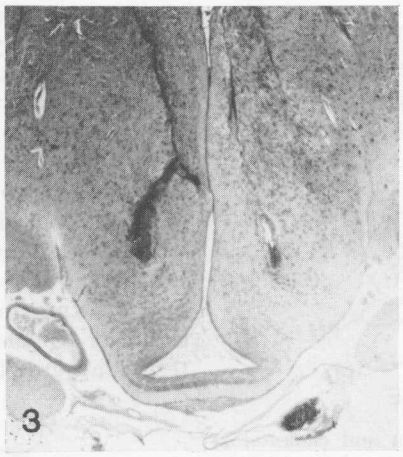


FIG. 2. The sites of puromycin and cholesterol (1:50) implants in the hypothalamic region 0.3–0.5 mm lateral to the third ventricle. (a) Effect on testicular growth. ○, An implant inducing no effect; ◐, an implant having a moderate effect on testicular growth; ●, an implant having a stronger effect. (b) Effect on development of the cloacal protrusion. ○, An implant having no effect; ◐, an implant having weak inhibition on cloacal development; ●, implant inducing stronger effect. Abbreviations are same as in Fig. 1.



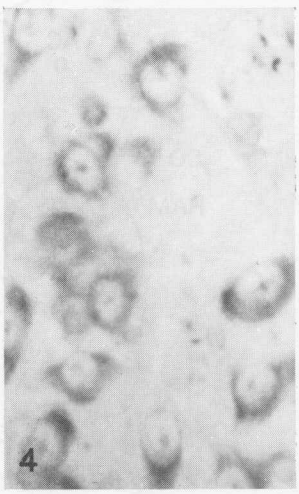


FIG. 3. Micrograph of ventral hypothalamus with bilateral puromycin (1:50) implants in the nucleus tuberis. The implant caused delayed development of the testes and the cloacal protrusion. ×35.

FIG. 4. Micrograph of neurons in the nucleus tuberis from just below the top of a tube containing cholesterol. ×700.

FIG. 5. Micrograph of neurons in the nucleus tuberis from just below the tip of a tube containing a mixture of puromycin and cholesterol (1:50). Note that nuclear diameters are small and the amount of cytoplasm is reduced in these neurons compared to those of the cholesterol control birds (Fig. 4). ×700.

cloacal protrusion was normal in one bird and inhibited in the other.

In Group 9, the tips of the implants were placed in the dorsal peripheral regions of the nucleus tuberis. One quail in this group had the tips in the anterior peripheral part of the nucleus tuberis and had delayed development of both organs. Four birds bearing the implants in the posterior peripheral regions of the nucleus tuberis demonstrated normal development of the testes, but delayed growth of the cloacal protrusions. Development of the testes and the cloacal protrusion was unaffected in the remaining five birds which bore the implants in the middle part of the peripheral regions of the nucleus tuberis.

The implants in areas other than the nucleus tuberis (Group 10), including the dorsal basal hypothalamus, did not have any effect on testicular and cloacal growth (Table 1 and Fig. 2a,b).

Histological observations of the hypothalamus showed that the neurons in the

regions just under the tips of the tubes ccntaining puromycin of this concentration had smaller nuclei and less cytoplasm than those near the cholesterol implants (Figs. 4 and 5).

Puromycin implants (1:50) in the hypothalamus tended to inhibit increase of body weight (significant in Group 10, see Table 1). Moreover, implants in the anterior nucleus tuberis and the peripheral regions of the nucleus tuberis caused the adrenals to increase in weight compared with the cholesterol birds.

DISCUSSION

A mixture of puromycin and cholesterol (1:50) implanted in the nucleus tuberis region induced delayed development of the testes and cloacal protrusion. These findings suggest that the synthesis of proteins or polypeptides including the gonadotropin releasing factor (GRF) was inhibited by puromycin and that the nucleus tuberis is the site of GRF production. The lower concentration of puromycin and cholesterol (1:500) did not affect the development of the testes and the cloacal protrusion. Several investigators have shown that lesions in the ventral hypothalamus prevent gonadal development in the White-crowned Sparrow (Wilson, 1967; Stetson, 1969a), in the chicken (Graber *et al.*, 1967), and in the Japanese quail (Sharp and Follett, 1969; Stetson, 1969b, 1972a,b). These results also suggest that the nucleus tuberis may be the site of GRF production, since the areas lesioned effectively in those experiments include the nucleus tuberis.

The nucleus tuberis, here identified on the basis of studies by van Tienhoven and Juhász (1962), seems to be identical to the basal layer of the nucleus infundibularis studied by Oehmke (1968, 1969, 1971). This basal layer of the nucleus infundibularis in the passerine birds (Oehmke, 1969; Oehmke *et al.*, 1969) and the nucleus tuberis of the quail (Sharp and Follett, 1968, 1970) contain the neurons with positive monoamine fluorescence. The basal layer of the nucleus infundibularis also contains the neurons which are not monoamine fluorescent (Oksche *et al.*, 1970, 1972). Oksche *et al.* (1972) have shown by morphometric techniques that the latter neurons can be activated by photostimulation. These results suggest that the nonmonoaminergic neurons in the nucleus tuberis are the site of GRF production. This idea is further supported by the fact that gonadotropin-releasing activity in the basal hypothalamus of the photostimulated quail is higher than that of nonphotostimulated quail (Follett, 1970). GRF production seems to be inhibited by testosterone since the implantation of this steroid into the nucleus tuberis inhibits testicular growth in the tree sparrow, *Spizella arborea* (Wilson, 1970; Cusick and Wilson, 1972) and in the Japanese quail (Wada, 1972; Stetson, 1972c).

Graber *et al.* (1967) showed that in the cockerel lesions destroying the posterior part of the tuberal nucleus or the tract proceeding to the posterior part of the anterior median eminence effected the interstitial cells of the testes and inhibited comb growth. They suggested that the posterior part of the tuberal nucleus might be involved in LH secretion. More recently, Stetson (1969b, 1972a,b) has demonstrated by lesioning experiments that in the quail the anterior portion of the basal hypothalamus (infundibular nuclear complex) controls the release of FSH and the posterior portion controls the release of LH. This is based on the finding that destruction of the anterior portion induced regression of the seminiferous tubules in males and regression of the ovary and oviduct in females, and destruction of the posterior portion induced regression of both gametogenic and endocrine components of the testes in males and cessation of ovulation in females. The present results support the idea that the posterior part of the nucleus tuberis controls the release of LH, because puromycin implanted in the posterior part of the nucleus tuberis did not inhibit the growth of the testes, but disturbed the development of the cloacal protrusion. This means that in these testes spermatogenesis, which is under the control of FSH, was not inhibited, but the activity of the interstitial cells, which is regulated by LH, was in-

hibited. It has also been suggested that testosterone implants in the posterior part of the nucleus tuberis effect only LH secretion as revealed by moderate inhibition of testicular growth and severe inhibition of the cloacal protrusion and seminal sac (Wada, 1972; Stetson, 1972c; Cusick and Wilson, 1972).

Sharp and Follett (1969) showed by lesioning studies that two areas, the nucleus tuberis and the nucleus in the dorsal basal hypothalamus around the paraventricular organ, are required to be intact for photostimulation to induce testicular growth in the quail. The present study, however, shows that the nucleus tuberis is the only area which is necessary for testicular growth. The nucleus in the dorsal basal hypothalamus, which is identical to the nucleus hypothalamic inferior in this presentation, does not seem to be involved directly in testicular development (Table 1, Group 10; Fig. 2a,b). Sharp (1972) showed that pituitary implants in the dorsal basal hypothalamus failed to maintain their glandular activity, whereas implants in the nucleus tuberis could maintain their glandular activity. From these findings, it seems safe to conclude that the site of GRF production is not the nucleus in the dorsal basal hypothalamus, but the nucleus tuberis. The neurons of the nucleus in the dorsal basal hypothalamus possibly stimulate the production of GRF in the nucleus tuberis.

The body weight decrease and the increase in adrenal weight induced by implantation of puromycin (1:50) in the hypothalamic regions are not clearly explicable at the present time.

ACKNOWLEDGMENTS

I wish to express my gratitude to Professor Hideshi Kobayashi for his valuable guidance during the course of this study. I also thank Professor Ikuo Yasumasu (Waseda University) for supplying the puromycin dihydrochloride and Professor C. D. Turner for reading the manuscript. This investigation was supported in part by a Grant from the Japan Society for the Promotion of Science, and Grant No. M67.133 from the Population Council to Professor S. Ishii, Waseda University.

REFERENCES

Asai, T., Uemura, H., and Kobayashi, H. (1969). Double staining for ordinary neurons and Gomori-positive neurosecretory system. *Zool. Mag.* (*Dobutsugaku Zasshi*) **78**, 108–111.

Cusick, E. K., and Wilson, F. E. (1972). On control of spontaneous testicular regression in tree sparrow (*Spizella arborea*). *Gen. Comp. Endocrinol.* **19**, 441–456.

Follett, B. K. (1970). Gonadotropin-releasing activity in the quail hypothalamus. *Gen. Comp. Endocrinol.* **15**, 165–179.

Graber, J. W., Frankel, A. I., and Nalbandov, A. V. (1967). Hypothalamic center influencing the release of LH in the cockerel. *Gen. Comp. Endocrinol.* **9**, 187–192.

Oehmke, H.-J. (1968). Regionale Strukturersheide im Nucleus infundibularis der Vögel (Passeriformes). *Z. Zellforsch. Mikrosk. Anat.* **92**, 406–421.

Oehmke, H.-J. (1969). Topographische Verteilung der Monoaminfluoreszenz im Zwischenhirn-Hypophysensystem von *Carduelis chloris* und *Anas platyrhynchos*. *Z. Zellforsch. Mikrosk. Anat.* **101**, 266–284.

Oehmke, H.-J. (1971). Vergleichende neurohistologische Studien am Nucleus infundibularis einiger australischer Vögel. *Z. Zellforsch. Mikrosk. Anat.* **122**, 122–138.

Oehmke, H.-J., Priedkalns, J., Harnack, V., and Oksche, A. (1969). Fluoreszenz und elektronmikroskopische Untersuchungen am Zwischenhirn-Hypophysensystem von *Passer domesticus*. *Z. Zellforsch. Mikrosk. Anat.* **95**, 109–133.

Oksche, A., Oehmke, H.-J., and Farner, D. S. (1970). Weitere Befunde zur Struktur und Funktion des Zwischenhirn-Hypophysensystem der Vögel. *In* "Aspects of Neuroendocrinology" (W. Bargmann and B. Scharrer, eds.), pp. 261–273. Springer-Verlag, Berlin and New York.

Oksche, A., Zimmermann, P., and Oehmke, H.-J. (1972). Morphometric studies of tuberoeminential systems controlling reproductive function. *In* "Brain-Endocrine Interaction. Median Eminence: Structure and Function" (K. M. Knigge, D. E. Scott, and A. Weindl, eds.), pp. 142–153. Karger, Basel.

Sachs, B. D. (1969). Photoperiodic control of reproductive behavior and physiology of the male Japanese quail (*Coturnix coturnix japonica*). *Horm. Behav.* **1**, 7–24.

Saxena, R. N., Wada, M., and Kobayashi, H. (1972). The implantation of puromycin into the neurosecretory nuclei of the rat. *Z. Zellforsch. Mikrosk. Anat.* **134**, 327–335.

Sharp, P. J. (1972). Pituitary implants in the hy-

pothalamus of *Coturnix* quail. *J. Endocrinol.* **53**, 329–330.

SHARP, P. J., AND FOLLETT, B. K. (1968). The distribution of monoamines in the hypothalamus of the Japanese quail, *Coturnix coturnix japonica*. *Z. Zellforsch. Mikrosk. Anat.* **90**, 245–262.

SHARP, P. J., AND FOLLETT, B. K. (1969). The effect of hypothalamic lesions on gonadotropin release in Japanese quail (*Coturnix coturnix japonica*). *Neuroendocrinology* **5**, 205–218.

SHARP, P. J., AND FOLLETT, B. K. (1970). The adrenergic supply within the avian hypothalamus. *In* "Aspects of Neuroendocrinology" (W. Bargmann and B. Scharrer, eds.), pp. 93–103. Springer-Verlag, Berlin and New York.

STETSON, M. H. (1969a). The role of the median eminence in control of photoperiodically induced gonadal growth in the White-crowned Sparrow, *Zonotrichia leucophrys gambelii*. *Z. Zellforsch. Mikrosk. Anat.* **93**, 369–394.

STETSON, M. H. (1969b). Hypothalamic regulation of FSH and LH secretion in male and female Japanese quail. *Amer. Zool.* **9**, 1078–1079.

STETSON, M. H. (1972a). Hypothalamic regulation of testicular function in Japanese quail. *Z. Zellforsch. Mikrosk. Anat.* **130**, 389–410.

STETSON, M. H. (1972b). Hypothalamic regulation of gonadotropin release in female Japanese quail. *Z. Zellforsch. Mikrosk. Anat.* **130**, 411–428.

STETSON, M. H. (1972c). Feedback regulation of testicular function in Japanese quail: Testosterone implants in the hypothalamus and adenohypophysis. *Gen. Comp. Endocrinol.* **19**, 37–47.

VAN TIENHOVEN, A., AND JUHÁSZ, L. P. (1962). The chicken telencephalon, diencephalon, and mesencephalon in stereotaxic coordinates. *J. Comp. Neurol.* **118**, 185–197.

WADA, M. (1972). Effect of hypothalamic implantation of testosterone on photostimulated testicular growth in Japanese quail (*Coturnix coturnix japonica*). *Z. Zellforsch. Mikrosk. Anat.* **124**, 507–519.

WILSON, F. E. (1967). The tubero-infundibular neuron system: A component of the photoperiodic control mechanism of the White-crowned Sparrow, *Zonotrichia leucophrys gambelii*. *Z. Zellforsch. Mikrosk. Anat.* **82**, 1–24.

WILSON, F. E. (1970). The tubero-infundibular region of the hypothalamus: A focus of testosterone sensitivity in male tree sparrow (*Spizella arborea*). *In* "Aspects of Neuroendocrinology" (W. Bargmann and B. Scharrer, eds.), pp. 274–286. Springer-Verlag, Berlin and New York.

ZAMBRANO, D., AND DE ROBERTIS, E. (1967). Ultrastructural aspects of the inhibition of neurosecretion by puromycin. *Z. Zellforsch. Mikrosk. Anat.* **76**, 458–470.