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Yolk is a source of maternal testosterone for developing birds

(egg/steroid hormone/embryo/sexual differentiation/aggression)

HUBERT SCHWABL

The Rockefeller University Field Research Center for Ecology and Ethology, Tyrrel Road, Millbrook, NY 12545

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ABSTRACT **The sex steroid hormones that affect development in birds have been thought to be produced exclusively by the embryo or neonate. I used radioimmunoassay to measure the amounts of androstenedione, 5α-dihydrotestosterone, testosterone, 17β-estradiol, and corticosterone in the yolk of freshly laid canary (*Serinus canaria*) and zebra finch (*Poephila guttata*) eggs. Testosterone was found in both canary and zebra finch eggs, but its contents were much higher in the former than in the latter. The testosterone content of canary eggs in a same clutch increased with the order of laying, regardless of the genetic sex of the offspring that hatched from these eggs. Yolk testosterone was also present in the eggs of female canaries that were kept without a male, indicating that it is of maternal origin. The social rank of juvenile canaries was positively correlated with the concentration of yolk testosterone in the eggs from which they hatched, suggesting that the development of aggressive behavior of offspring might be subject to modification by maternal testosterone. These findings indicate that female songbirds can bestow upon their eggs a dose of hormone that modifies the behavior of offspring. Variable doses of these hormones might explain some of the individual variation in offspring behavior.**

Development of sex in higher vertebrates occurs in two steps. First the genotype determines the production of ovaries or testes. Then differential secretion of sex hormones determines the development of male and female secondary sex characters and behavior (1–4). Earlier studies of sex hormones in sexual differentiation (5–10) assumed that in birds, in which the embryo develops in the egg outside the mother, the developing organism was the main source of sex hormones that influence development. However, steroid hormones are lipophilic, and they may pass from the mother into the lipoprotein matrix of egg yolk during vitellogenesis. The presence of such hormones might affect developmental processes before embryonic steroidogenic tissue becomes active and could influence the fitness of offspring. That steroid hormones can pass from the female's circulation into the egg is suggested by experiments that show modification of offspring sexual differentiation in response to treatment of the egg-laying female with exogenous estradiol (11, 12). Indirect evidence for the presence of steroid hormones with estrogenic activity in egg yolk comes from bioassays (reviewed in ref. 13). However, except for a chromatographic investigation of chicken egg yolk that failed to demonstrate E_2 (13), the hormone contents of the eggs of untreated birds have not been systematically investigated. I used radioimmunoassay to measure the amounts of sex steroid hormones and corticosterone in freshly laid eggs of canaries and zebra finches.

MATERIALS AND METHODS

Canary and Zebra Finch Eggs Used for Initial Assays of Steroid Content. Eggs were obtained on the day of laying from canaries of the Belgian Waterslager strain during the 1992 breeding season and from zebra finches during 1991. Canaries were housed under a photoperiod that corresponded to that of New York State (42°N). Zebra finches were housed under a light regimen of 12 hr of light and 12 hr of darkness. Temperature was maintained at 20°C. Food and water were available ad libitum. Eggs were dissected on the day they were laid and the yolk was separated and frozen.

Eggs Used To Assay the Variation of T Content Within Canary Clutches. Ten pairs of canaries were transferred in November from the simulated natural photoperiod of New York State (42°N) to a light regimen of 8 hr of light and 16 hr of darkness. After 21 days, the light time was increased to 14 hr to stimulate reproduction, and nesting cups and nesting material were offered. Nests were checked daily for eggs after the lights went on. Females started to lay 14 days after photostimulation started. Eggs were removed, and a small yolk sample (10–15 mg) was obtained by inserting a 25-gauge needle through the small end of the egg into the yolk. After sealing the hole with transparent wound dressing, the eggs were returned to the nests, so that clutch size was not changed. The yolk sample was weighed, diluted with 0.5 ml of distilled water, mixed, and frozen for hormone assays. In addition, eggs from three females that were kept without a male were removed daily from the nests during the 1992 breeding season. In this case eggs were not returned to the nest, and the yolk was collected and frozen. Eggs laid at intervals of no more than 2 days were considered to belong to the same clutch.

Steroid Hormone Assays. *Initial assays of hormone contents of canary and zebra finch yolk.* Yolks were thawed and homogenized in 1 ml of distilled water by a vigorous mixing facilitated by the addition of a few glass beads. Tritiated steroid hormones (2000 cpm per zebra finch and 10,000 cpm per canary egg yolk) were added to the homogenate for calculation of extraction and purification recoveries. After further mixing, the homogenate was allowed to equilibrate for at least 24 hr at 4°C. Free steroids were then extracted twice with 3 ml of petroleum ether/diethyl ether, 30:70 (vol/vol). The ether fractions were decanted from the snap-frozen egg yolk/water phase, combined, and dried under a stream of nitrogen. The dried gross extract was redissolved in 1 ml of 90% ethanol and kept overnight at −20°C. Precipitated proteins and lipids were separated from the ethanol phase by decanting after centrifugation at 1300 × *g* for 5 min. The ethanol phase was washed with 2 ml of hexane to further remove lipids. The hexane phase was washed with 1 ml of 90% ethanol, and the two ethanolic phases were combined and dried under nitrogen. The dried extract was redissolved in 1 ml of 10% (vol/vol) ethyl acetate in 2,2,4-trimethylpentane. The entire 1 ml of the zebra finch yolk extract or 50 μl of the canary yolk extract was transferred onto a diatomaceous earth microcolumn. Removal of lipids and partial purification of steroid hormones on these columns and the following radioimmunoassays were by published methods

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Abbreviations: A_4, androstenedione; DHT, 5α-dihydrotestosterone; T, testosterone; E_2, 17β-estradiol.

(14, 15). Recoveries were as follows: androstenedione (A_4), 53%; 5α-dihydrotestosterone (DHT), 59%; testosterone (T), 62%; 17β-estradiol (E_2), 72%; and corticosterone, 44%. The lower detection limits for A_4, DHT, T, E_2, and corticosterone were 4, 8, 8, 4 and 8 pg per tube.

Assays of hormone concentration of canary eggs within a clutch. The yolk sample was thawed and transferred to a glass extraction vial. After addition of 2000 cpm of tritiated hormone, samples were extracted and assayed as described above.

Assays of hormone concentration of unfertilized canary eggs. Frozen yolk from single females was thawed, and a weighed amount of about 20 mg was dissolved in 0.5 ml of distilled H_2O and processed as described for biopsy samples.

Validation of Hormone Assays. *Specificity and accuracy.* Endogenous steroids were removed by treatment of yolk with 1 ml of charcoal solution (10 mg/ml). Known amounts of steroid hormones were then added. After equilibration for 24 hr, steroids were extracted and subjected to radioimmunoassay. The results suggest that nonspecific interference of egg yolk substances with binding to the antibody in the radioimmunoassays is absent and that the respective hormones can be measured in quantitative amounts (Table 1). This is further indicated by parallelism of dilution curves of egg yolk with dilution curves of the respective steroid hormones. The antisera used in the assays cross-react slightly with other steroids. However, it is unlikely that some of these steroids, if present in egg yolk, could have cross-reacted with the respective steroid hormone assays because of their separation from the respective fraction during chromatography prior to assays (15).

Mass spectrometry. Mass spectrometry of an extract of canary egg yolk confirmed the identification of T in radioimmunoassays. Appropriate retention time and a double peak in the chromatogram identified T in canary yolk. Hence, it is reasonable to accept that the activity detected by radioimmunoassay is identical to testosterone. It was not possible to identify A_4, DHT, E_2, and corticosterone by mass spectrometry, probably because they were present only in low amounts.

Behavioral Investigation. Fledglings were separated from their parents 20–25 days after they had hatched and kept in groups of three siblings. Perhaps as a result of the biopsies, not more than three siblings were produced by any of the clutches, and smaller groups were not investigated. The five investigated sibling groups resulted from clutches used in the biopsy experiment. Sex was identified by visual inspection of the gonads via unilateral laparotomy between 28 and 30 days of age, and group composition was varied as follows: three groups were composed of one male and two females, one group was all male, and one group was all female. Juveniles from the same clutch were housed in the same cage under a photoperiod of 14 hr of light and 10 hr of darkness. At the time of behavioral testing, birds were between 46 and 131 days old. The social rank was determined by placing a single food dish on the floor of the cage after 2 hr of food deprivation. Order of access to the food dish and aggressive interactions were observed for 20 min. Both the order in which the birds started to feed and the frequency with which individuals supplanted each other from the dish were used to rank the birds.

Statistical Analyses. Variation of T content of eggs within a clutch was analyzed by ANOVA for repeated measures followed by *t* tests of paired samples for comparisons among eggs within a clutch. T contents of eggs from which juveniles of different social rank hatched were compared by the Wilcoxon signed ranks test.

Table 1. Amounts of A_4, DHT, T, E_2, and corticosterone (B) measured by RIA after addition to ligand-free egg yolk

Hormone addition to yolk, pg	Hormone measured in yolk after addition, pg				
	A_4	DHT	T	E_2	B
0	ND (2)	ND (2)	ND (4)	ND (4)	ND (4)
100	—	—	127 (2)	120 (2)	—
250	—	—	237 (3)	—	219 (3)
500	—	—	554 (5)	466 (6)	452 (8)
1000	891 (2)	1277 (2)	—	—	993 (5)
2500	—	—	2676 (2)	2669 (2)	—

The number of samples for each measurement are given in parentheses. Hormones were added in 0, 100-pg, 250-pg, 500-pg, 1000-pg, and 2500-pg amounts and allowed to equilibrate before measurement by RIA. ND, not detectable.

RESULTS

Presence of Sex Steroid Hormones in Canary and Zebra Finch Eggs. A_4, DHT, and T were found in the yolk of all canary eggs (Fig. 1). T was present in lower amounts also in zebra finch eggs; A_4 and DHT were not analyzed in this species. E_2 was detectable (>0.184 ng) in 59% of the canary eggs and in 27% of the zebra finch eggs (>0.05 ng). Corticosterone was undetectable (<0.5 ng) in all 29 canary eggs and was detected at low levels in 26 of 27 zebra finch eggs (<0.1 ng, data not shown in Fig. 1).

The yolk of canary eggs contained significantly more T than that of zebra finches (Mann–Whitney *U* test; $P < 0.001$). This was not due to the larger amount of yolk of canary than zebra finch eggs. While canary eggs contained about twice as much yolk as those of zebra finches, T content was approximately 7 times higher in canary than in zebra finch yolk (Fig. 1). The amount of none of the hormones assayed was correlated, within species, with yolk mass.

Variation of T Content of Eggs Within Canary Clutches. The T content of canary eggs varied over a wide range (Fig. 1). One source of this variation was the order in which eggs were laid in a clutch. I analyzed the T concentration in the eggs of successive clutches of seven pairs of canaries. The first egg in each clutch had lower T content than later eggs, with contents gradually rising between the first and third egg. This order effect was clearly discernable in up to five successive clutches that were laid by some of the females (Fig. 2). Comparable high amounts of T were also measured in eggs laid by females that were kept without a male (Fig. 3). In this case eggs in positions 2–5 had higher levels than eggs in position 1, although no statistical statement about the difference can be made because only three females were involved and some laid more eggs than others.

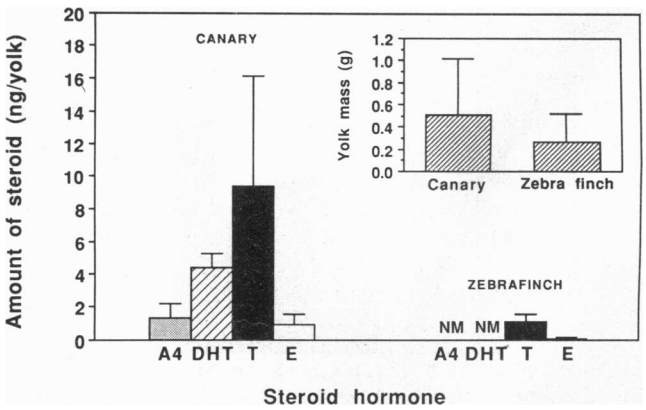

FIG. 1. Amounts (means ± SD) of A_4, DHT, T, and E_2 per yolk of freshly laid canary and zebra finch egg. A_4 and DHT contents were not measured (NM) in zebrafinch eggs. (*Inset*) Mean yolk mass with standard deviation of the 29 canary and 27 zebra finch eggs that were analyzed.

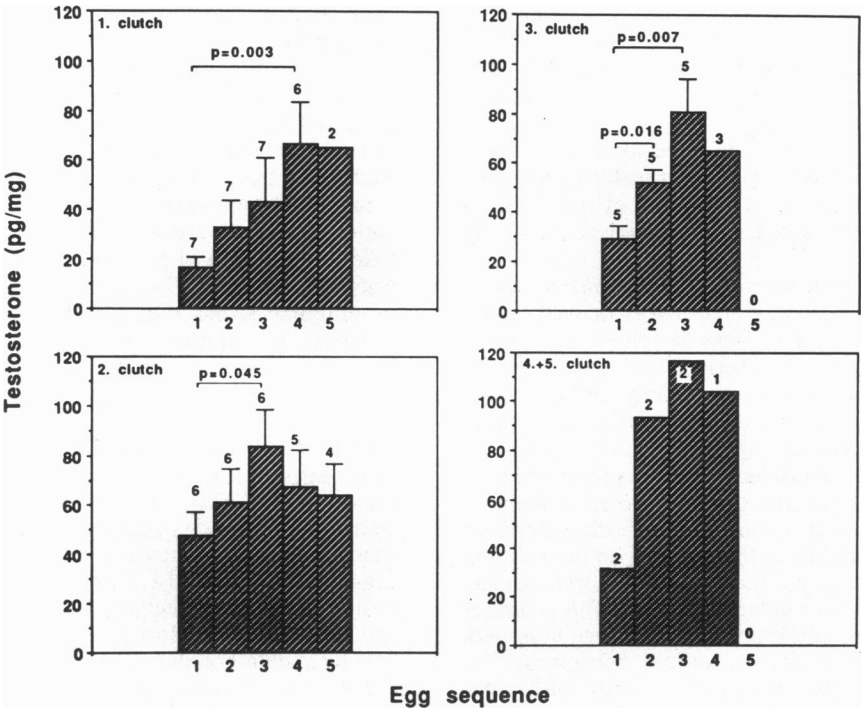

FIG. 2. T concentration (in picograms per mg of yolk; means ± SEM) of eggs from successive clutches laid by seven pairs of canaries, showing the order of laying in a clutch. Numbers on top of columns indicate numbers of eggs. T levels varied with order of laying (clutch 1: $F = 4.92$, $P = 0.019$; clutch 2: $F = 6.71$, $P = 0.011$; clutch 3: $F = 0.69$, $P = 0.54$). Statistical analysis was not done on clutches 4 and 5, here shown together, because of the small number of eggs laid. Significant differences in T contents between eggs are indicated by brackets.

Both males and females hatched from eggs with low or high T contents (Fig. 4).

Behavioral Effects of Yolk T. The social rank of sibling canaries was correlated with the amount of T they were exposed to in the egg (Fig. 5*A*). Both the order (1–3) in which birds started to feed after food deprivation and the frequency with which individuals supplanted each other from the food dish resulted in the same social hierarchy of high (1), intermediate (2), and low (3) rank. Thus, the social rank that was assigned to each individual bird reflects the order of access to a single food source as well as the dominance of one bird over another one by aggression. The positive correlation of the social rank of juveniles with the amount of T in the eggs from which these birds had hatched appears to apply to both males and females (Fig. 5*B*).

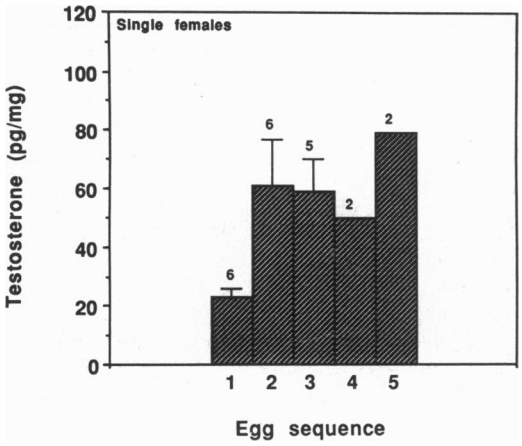

FIG. 3. T concentrations (picograms per mg of yolk; means ± SEM) of eggs laid by three female canaries kept without a male as a function of the order in which eggs were laid. Numbers above columns indicate numbers of eggs in each position.

DISCUSSION

The present study demonstrates that avian egg yolk contains maternal sex steroid hormones, in particular T. The presence of high amounts of T in the yolk could provide an explanation for results from bioassays that demonstrated estrogenic properties of yolk (reviewed in ref. 13) when one takes into account that in target tissues T can be aromatized to estrogens.

Canary yolk contained more T than zebra finch yolk, which is not explained by the difference in yolk mass. I have no explanation for this difference; the ecological, behavioral, and developmental significance of species differences in yolk hormones should be studied. The rest of this discussion focuses on T in the canary egg.

In the canary yolk, T content increased with order of laying in a clutch. The observation that also the eggs of females that laid without access to a male had high T contents rules out the

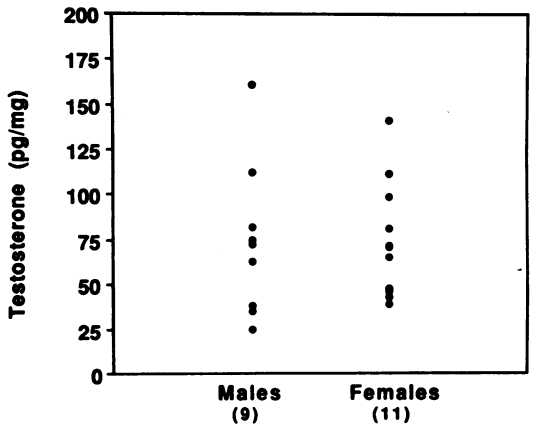

FIG. 4. T concentrations (picograms per mg of yolk) of canary eggs from which males or females hatched.

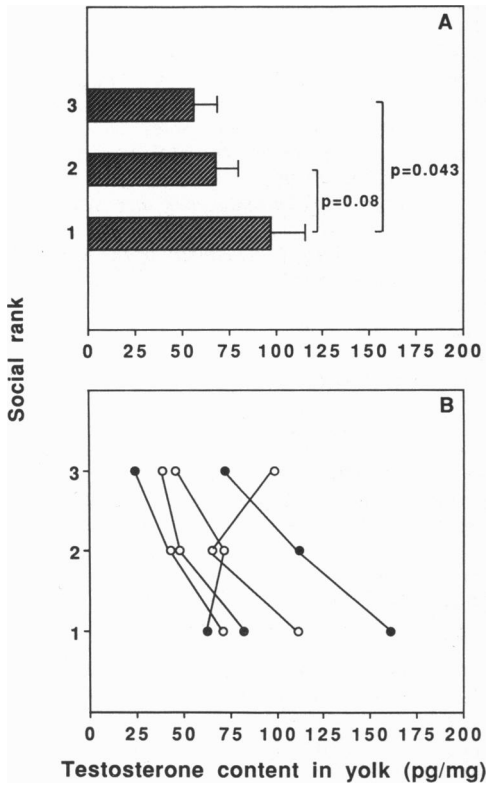

FIG. 5. (*A*) Concentration of maternal T (means ± SEM) measured in the eggs from which sibling juvenile canaries of different social rank hatched. High (1), intermediate (2), and low (3) social rank were assigned from observations of access to food. Levels of significance of T egg concentrations between birds of different ranks are indicated next to brackets. (*B*) T concentrations of the eggs and the social rank of the individual birds of each of these cohorts. Note the variable composition of the groups of males (●) and females (○).

possibility that fertilization or the transfer of T from the male to the egg during copulation caused the high T levels that were observed. Thus, the T must be incorporated into the yolk in the ovary and the increasing contents of it in subsequent eggs of a clutch may result from the ovarian follicular hierarchy, yolk formation, and sequential ovulation of each of the eggs as described below.

Yolk is laid down in the follicle during its rapid-growth phase, which begins between 5 and 9 days before ovulation of the follicle, depending on the species (16). In chicken blood, T levels start to rise around 8 hr before ovulation of the follicle and peak 4–5 hr before ovulation; 24 hr after ovulation, the egg is laid, and within 15–75 min the next follicle ovulates (17). Thus, whereas the egg laid first is subjected only to the T surge that occurs prior to its ovulation, the subsequently laid eggs are also subjected to the surges that preceded ovulation of each of the earlier eggs in the clutch. If increased exposure to T is accompanied by diffusion or active transport of T from the blood into the egg yolk, then we would have here the mechanism responsible for the increase in yolk T with order of laying. Female birds produce male and female follicles (18). Yet these two kinds of follicles do not seem to accumulate T into their yolk in a differential manner, since male and female embryos developed in eggs that were distributed over the entire range of yolk T contents. The increase in yolk T with order of laying was particularly marked and reliable for the first three eggs. Therefore, incorporation and retention of T may occur only during the 3 days before ovulation. In addition, the presence of a male may influence T production or incorporation into the yolk, since the correlation of yolk T with order of laying was not as clear in single females as in mated females.

Embryos assimilate yolk heavily during the second half of incubation (19), and so exposure to maternal T may be particularly high during this time. One process that is likely to be influenced by T is growth. Neurons of the spinal cord and the glycogen body, an organ for polysaccharide storage within the vertebral column, are targets of androgens as early as day 10 of incubation in both male and female chicken embryos (20). Moreover, androgens induce neurite growth in the spinal cord of fetal mice, and this effect is not restricted to segments that are sexually dimorphic (21). These earlier observations suggest that the overall growth of the neuromuscular systems of both sexes may be enhanced by T. If higher yolk T contents result in faster growth of the motor system of embryos that develop from eggs laid late in a clutch, then these younger individuals in a clutch might be compensated, to some extent, for the disadvantage of having to compete with older siblings in the nest. In the canary, incubation starts before the last egg is laid and results in the asynchronous hatching of eggs usually 1 day apart as in other songbird species (22, 23).

The differentiation of primary sex characteristics does not seem to be influenced by yolk T. Otherwise different sexes would hatch from eggs with low or high T contents, which is not the case. At present I cannot rule out that maternal yolk T influences the differentiation of secondary sex characteristics, including sex-specific brain pathways and behavior. Even if the behavioral differentiation of altricial songbirds, such as the canary, takes place mainly after hatching (24) when most of the yolk is spent and maternal T is no longer available to directly affect it, maternal T could have an effect—for example, by changing the sensitivities of target tissues to the embryo's own gonadal secretions later in life (25–27).

A more general developmental role of maternal T that is not related to sexual differentiation and sex-specific behavior is suggested by the positive correlation between the social rank of juveniles of both sexes and the T content of the eggs from which each bird hatched. The mechanism by which high content of T in the egg results in a high ranking individual regardless of sex could be by an anabolic effect on embryonic growth, as discussed above. However, brain functions that allow an individual to achieve higher rank (for example, aggression) could also have been modified in a specific manner by exposure to maternal T in the egg. Whatever the exact proximate mechanism may be, modification of developmental processes by maternal hormones could provide an explanation for some of the great inter-individual variation in aggressive behavior that is so far unexplained.

The theory of optimal reproductive investment identifies the number and size of gametes as variables (28). Here I suggest as an additional variable egg quality other than the amount of allocated nutrient reserves (e.g., refs. 29 and 30)—namely, females may bestow upon their eggs substances (e.g., hormones) that affect fitness by modification of the behavior of offspring. The present study suggests aggression as a possible offspring behavior that is modified by the amount of maternal T that is present in the egg.

Modification of offspring traits by maternal hormones has been shown in mammals. In these studies the role of the maternal hormonal state in development is difficult to assess because the fetus is exposed to the mother throughout gestation, and in cases with more than one offspring in a litter, there is also exposure to hormones from adjacent fetuses (e.g., refs. 31–34). The presence of such maternal effects in egg-laying vertebrates, in which all development takes place outside of the mother, provides an ideal model in which to study the contribution of the maternal hormonal state during

egg formation to phenotypic variation (35) in morphology, physiology, and behavior of offspring.

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1. Breedlove, M. S. (1992) in *Behavioral Endocrinology*, eds. Becker, J. B., Breedlove, M. S. & Crews, D. (MIT Press, Cambridge, MA), pp. 39–68.
2. Adkins-Regan, E. (1981) in *Neuroendocrinology of Reproduction: Physiology and Behavior*, ed. Adler, N. T. (Plenum, New York), pp. 159–221.
3. Feder, H. F. (1981) in *Neuroendocrinology of Reproduction: Physiology and Behavior*, ed. Adler, N. T. (Plenum, New York), pp. 89–118.
4. Feder, H. F. (1981) in *Neuroendocrinology of Reproduction: Physiology and Behavior*, ed. Adler, N. T. (Plenum, New York), pp. 127–151.
5. Tanabe, Y., Yano, T. & Nakamura, T. (1983) *Gen. Comp. Endocrinol.* **49,** 144–153.
6. Tanabe, Y., Saito, N. & Nakamura, T. (1986) *Gen. Comp. Endocrinol.* **63,** 456–463.
7. Schumacher, M., Sulon, J. & Balthazart, J. (1988) *J. Endocrinol.* **118,** 127–134.
8. Hutchison, J. B., Wingfield, J. C. & Hutchison, R. T. (1984) *J. Endocrinol.* **103,** 363–369.
9. Schlinger, B. A. & Arnold, A. P. (1992) *Endocrinology* **130,** 289–299.
10. Adkins-Regan, E., Abdelnabi, M., Mobarak, M. & Ottinger, M. A. (1990) *Gen. Comp. Endocrinol.* **78,** 93–109.
11. Riddle, O. & Dunham, H. H. (1942) *Endocrinology* **30,** 959–968.
12. Adkins-Regan, E. (1983) in *Hormones and Behaviour in Higher Vertebrates*, eds. Balthazart, J., Pröve, E. & Gilles, R. (Springer, Berlin), pp. 218–228.
13. Hertelendy, F. & Common, R. H. (1965) *Poult. Sci.* **44,** 1205–1209.
14. Wingfield, J. C. & Farner, D. S. (1975) *Steroids* **26,** 311–327.
15. Abraham, D. E. (1974) *Acta Endocrinol. Suppl.* **183,** 7–42.
16. Lehrman, D. (1961) in *Sex and Internal Secretions*, ed. Young, W. C. (Williams & Wilkins, Baltimore).
17. Shahabi, N. A., Norton, H. W. & Nalbandov, A. V. (1975) *Endocrinology* **96,** 962–968.
18. Ohno, S. (1967) *Sex Chromosomes and Sex-Linked Genes* (Springer, Berlin).
19. Romanoff, A. L. (1967) *Biochemistry of the Avian Embryo: A Quantitative Analysis of Pre-Natal Development* (Wiley, New York).
20. Reid, F. A., Gasc, J.-M., Stumpf, W. E. & Sar, M. (1981) *Exp. Brain Res.* **44,** 243–248.
21. Hauser, K. F. & Toran-Allerand, C. D. (1989) *Brain Res.* **485,** 157–164.
22. Lack, D. (1968) *Ecological Adaptations for Breeding in Birds* (Methuen, London).
23. Clark, A. B. & Wilson, D. S. (1981) *Q. Rev. Biol.* **56,** 253–277.
24. Adkins-Regan, E. (1987) *Trends Neurosci.* **10,** 517–522.
25. Elbrecht, A. & Smith, R. G. (1992) *Science* **255,** 467–470.
26. Balthazart, J., De Clerck, A. & Foidart, A. (1992) *Horm. Behav.* **26,** 179–203.
27. Adkins, E. K. (1976) *Physiol. Behav.* **17,** 357–359.
28. Stearns, S. (1992) *The Evolution of Life Histories* (Oxford Univ. Press, Oxford).
29. Sinervo, B. & Huey, R. B. (1990) *Science* **246,** 1106–1109.
30. Williams, T. D., Lank, D. B. & Cooke, F. (1993) *Oikos* **67,** 250–256.
31. Clemens, L. G., Gladue, B. A. & Coniglio, L. P. (1978) *Horm. Behav.* **10,** 40–53.
32. vom Saal, F. S. & Bronson, F. H. (1978) *Biol. Reprod.* **19,** 842–853.
33. Licht, P., Frank, L. G., Pavgi, S., Yalcinkaya, T. M., Siiteri, P. K. & Glickman, S. E. (1992) *J. Reprod. Fertil.* **95,** 463–474.
34. Evan, M. D., Dhar, M. G. & vom Saal, F. S. (1992) *J. Reprod. Fertil.* **96,** 709–716.
35. West-Eberhard, M. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1388–1392.