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Seasonal changes in plasma retinol-binding holoprotein concentration in Japanese quail (*Coturnix coturnix japonica*)

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1. Seasonal changes in retinol-binding holoprotein (holoRBP) concentration in plasma of groups of male and female Japanese quail (*Coturnix coturnix japonica*) were examined over 18 months.

2. In Expts 1 and 2 the birds were maintained under natural lighting conditions and in Expt 3 under artificial-light photoperiods corresponding to the changing daylength at 56° N latitude. All groups were at 18–20° and received Superlayers' (Rank Hovis McDougall) pellet diet.

3. The mean plasma holoRBP concentration in all groups changed in an annual cycle with minimal values in September–October and maximal values in February–April, when daylength or light photoperiod increased to more than 10 h.

4. The group mean values in the female cycle change 2- to 3-fold from 50–100 μg/ml in late summer to 220–280 μg/ml in the spring, whereas in the male the range is only 1·3–1·5 times, from 140–170 to 180–250 μg/ml.

5. In the female the rate of egg laying was maximal in April–May and lowest in November–December.

6. The spring increase in plasma holoRBP reflects the increased vitamin A requirement of birds for reproduction and it is presumably under hormonal control. The wider amplitude in the female cycle compared with the male probably arises from the additional demand for the transfer of vitamin A into the eggs and hence the need for a higher initial secretion rate from the female liver to meet it.

Retinol-binding protein (RBP) is the specific carrier protein for vitamin A in plasma. It is necessary for the distribution of the vitamin to target tissues such as the eye, epithelial tissues and the gonads. Retinol is bound to the protein (approximately 21000 daltons) in a 1:1 complex in the human (Kanai *et al.* 1968) and in all other animal species so far examined including the chick (Abe *et al.* 1975). This material is called holoRBP to distinguish it from the uncomplexed or unsaturated apoRBP. It has been established previously that the absence of vitamin A for completion of the synthesis of the bimolecular complex in the liver inhibits the release of the protein into the bloodstream by as much as 70% (Muto *et al.* 1972). Alternatively, deficiency of good-quality protein in the diet of an otherwise vitamin A-replete subject reduces the pool of some essential amino acids for protein biosynthesis in the liver and lowers the plasma RBP concentration by as much as 30% below control values (Muhilal & Glover, 1974). Thus the plasma level of RBP can change as a consequence of nutritional deficiencies. However, in the normal seasonal breeding animal like the sheep, replete with vitamin A and protein, the concentration of holoRBP undergoes annual cyclic changes from a minimum of 25 μg/ml in the summer to a maximum of 100 μg/ml plasma in the autumn and then gradually regresses in the spring to the summer value (Glover *et al.* 1976).

The autumnal surge in RBP concentration occurs in both sexes with decreasing daylength which has also long been known (Yeats, 1949) to effect changes in the sexual development of sheep. This relationship between RBP concentration and functioning of the gonads is consistent with the previous findings of Thompson *et al.* (1964) that retinol is specifically required for the proper functioning of the gonads in the rat. The cyclic or rhythmic changes in their functioning are mediated by the hypothalamo-pituitary hormonal axis, but the initiating photoreceptor systems for entrainment of animals (Wurtman *et al.*

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1968) and of birds (Murton & Westwood, 1977) to particular photoperiodic cycles are quite different. It was of interest, therefore, to determine how holoRBP concentration would change in birds which are sexually active in the spring. Japanese quail (*Coturnix coturnix japonica*) were used for the study since they have been used successfully for many experiments on reproduction and have a breeding cycle known to be influenced by photoperiod (Wilson *et al.* 1962). A preliminary report of the work has already been published (Glover & Large, 1977).

MATERIALS AND METHODS

Animals. Japanese quail were obtained from the Craven Game Farm, Clitheroe, Lancs. Groups of four male (M_1) and four female (F_1) (approximately 8 weeks old) were obtained in December 1975 for the preliminary Expt 1 and groups of five of each sex (M_2 and F_2) were used in Expt 2 commencing April 1976. They were maintained indoors at 18–20° under natural lighting conditions throughout. Precautions were also taken to screen the birds from exterior street lighting. They were placed on a diet of Starter crumbs initially and later on Superlayers' pellets (Rank Hovis McDougall) *ad lib.* The diet contained sufficient provitamin A and vitamin A to meet their needs.

In Expt 3, groups of nine male (M_3) and seven female (F_3) birds (3–6 months old) were maintained from 3 February 1977 under artificial light from daylight fluorescent tubes controlled by a solar dial clock (Sangamo Weston Ltd, Enfield, Middx) to simulate the natural photoperiod from sunrise to sunset corresponding to latitude 56° N throughout the year. These birds were maintained on Superlayers' pellets throughout the experiment. All birds were distinguished by appropriate colour coded plastic rings or placed in separate cages. A record of the eggs laid by group F_3 birds was kept from the time when they were placed in artificial light.

Methods. Blood was taken from a toe vein every 2 weeks in the late winter and spring, but only monthly thereafter in Expts 1 and 2. In Expt 3 blood was also taken fortnightly but from the wing vein. In Expts 1 and 2 all samples were collected directly into 100 μl EDTA-treated capillary tubes which were sealed at one end before centrifuging to separate the plasma. In Expt 3 the anticoagulant was heparin. Several tests showed that there was no difference in the concentration of holoRBP in plasma taken from either the toe or wing vein of an individual bird at the same time. The plasma was analysed for holoRBP by the fluorescence method as previously described (Glover *et al.* 1974). This involved separating the plasma proteins in a 10 μl sample using disc-gel electrophoresis. The gel was then scanned with a narrow beam of u.v.-light (Wood's glass) from a mercury lamp and the fluorescence emission in the 460 nm region recorded.

A typical scan of Japanese quail plasma is shown in Fig. 1 alongside that of human plasma. The area of the first peak is directly related to holoRBP concentration. It can be seen that the relative mobility of Japanese quail holoRBP is very similar to that of human RBP. The presence of retinol in the zone corresponding to the first peak of the fluorescence scan used for assay was confirmed by taking some quail plasma and separating out the holoRBP on an ion-exchange column. The fluorescence and excitation spectrum of this purified material was measured using a fluorescence spectrophotometer (Perkin Elmer Hitachi MPF 2A) and found to have similar characteristics to those of human and chick holoRBP as shown in Table 1.

The molecular weight of the pure RBP isolated from quail plasma has been determined in our laboratory to be approximately 20000 daltons (D. Boag, unpublished observations) and very similar to that of chicken RBP (Abe *et al.* 1975). Colorimetric assay of the retinol in holoRBP and immunoassay of the protein moiety confirmed that only 1 mol retinol binds to 1 mol protein. The factor for conversion of the retinol fluorescence double-peak

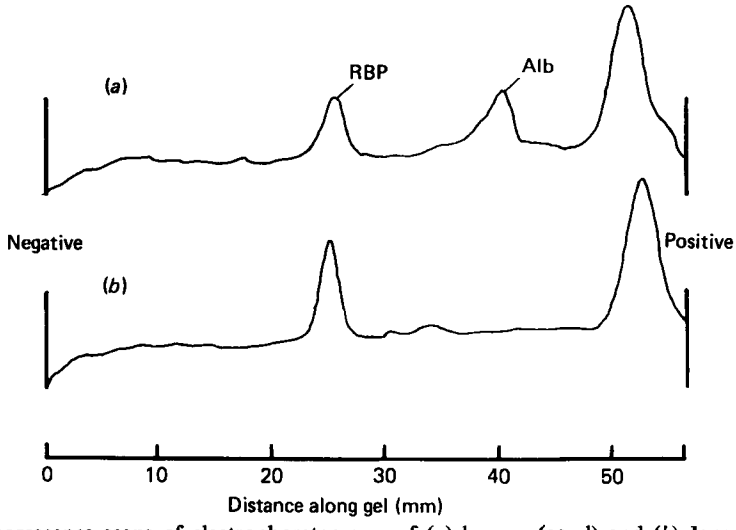

Fig. 1. Fluorescence scans of electrophoretograms of (*a*) human (20 μl) and (*b*) Japanese quail (*Coturnix coturnix japonica*) (10 μl) plasma on polyacrylamide (50 g/kg) disc-gels. RBP, retinol-binding protein; Alb, albumin.

Table 1. *Characteristics of yellow-green fluorescent protein in the α-globulin zone of polyacrylamide disc-gel electrophoretogram of Japanese quail* (Coturnix coturnix japonica) *plasma compared with those of pure chicken and human retinol-binding holoprotein*

	Japanese quail	Chicken	Human
Electrophoretic mobility relative to human albumin	0·65	0·64	0·66
Fluorescence excitation λ_{max} (nm)	338	334	332
Fluorescence emission λ_{max} (nm)	460	456	456

areas in the scan of the gel to holoRBP concentration in plasma was found to be 0·3 for both chicken and Japanese quail and very similar to that (0·29) used for human holoRBP using the same instrument. The error for sets of analyses carried out at the same time was < 3 % as previously reported, but the interassay error between sets carried out at different times over the year was a little larger at 7 % based on the analyses of standard serum samples maintained at < −20° throughout the period of the experiments. The results for Expt 3, however, have been corrected to eliminate the interassay error.

Statistics. In order to examine general trends in plasma holoRBP concentration, the mean values for determinations within short periods in the cycle were compared with those for succeeding periods using the paired *t* test.

RESULTS

The mean plasma values with their standard errors for holoRBP concentration in the various groups of male and female birds are plotted *v.* time (weeks) over the period of the experiment in Fig. 2 (Expt 1 and 2 with natural light) and Fig. 3 (Expt 3 with artificial light). The calendar months are also indicated. It is clear from Figs 2, 3 that minor oscillations in mean plasma holoRBP concentration occur throughout the year in addition to major variations. Consequently, in an attempt to show up the general trends over the

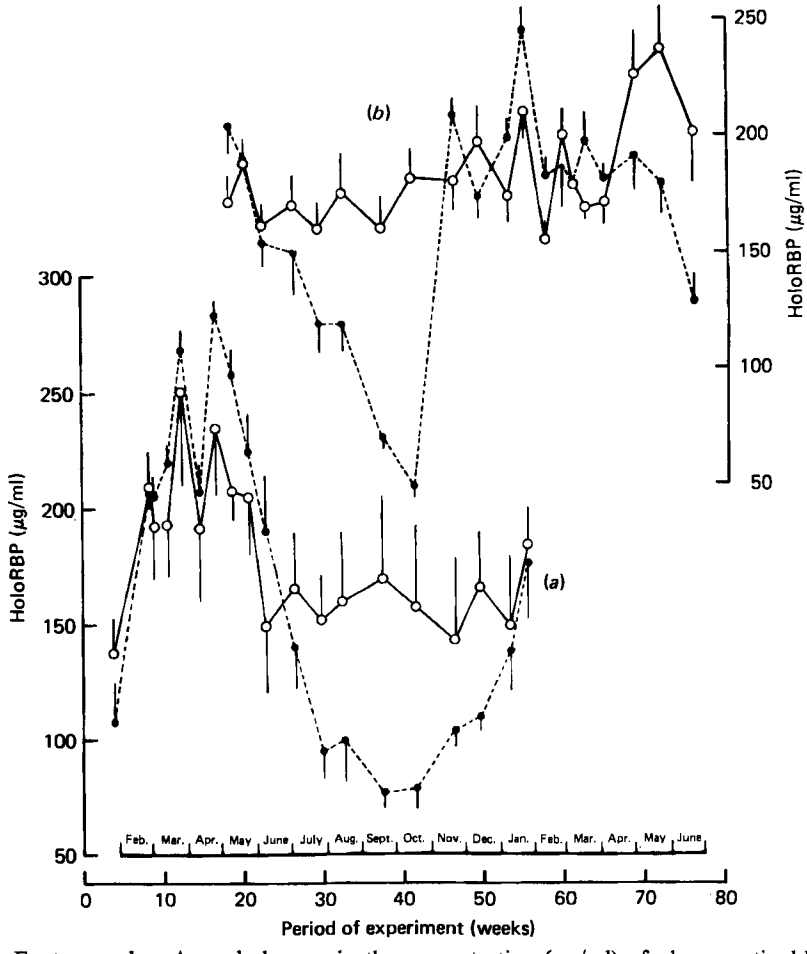

Fig. 2. Expts 1 and 2. Annual changes in the concentration (μg/ml) of plasma retinol-binding holoprotein (holoRBP) in groups of male (○—○) and female (●---●) Japanese quail (*Coturnix coturnix japonica*) maintained under natural lighting over the period January 1976 to June 1977. (*a*) Expt 1; (*b*) Expt 2; for details of experiments see p. 358. Points represent mean values with their standard errors represented by vertical bars.

year 'interval' mean values were determined for each group over 2 month intervals from the beginning of the breeding season in February to July and over 3 month periods in the sexually quiescent phase from August to January. The results are set out in Table 2. The results for January only at the commencement of Expt 1 and for June at the end of Expts 2 and 3 have been treated separately. The change in holoRBP concentration from one period to the next and its level of significance (*P* value) are also inserted. Peak values are highlighted by insertion of a bar above the figures and minimal values with a bar below them. In Expt 1, since the general changes in plasma holoRBP concentration in this M_1 group followed closely those for F_1 in the same experiment both sets of results were taken together for statistical treatment.

To consider firstly the female groups F_1 to F_3, the results in Figs 2, 3 and Table 2 show that plasma holoRBP concentration was minimal in the August to October period with

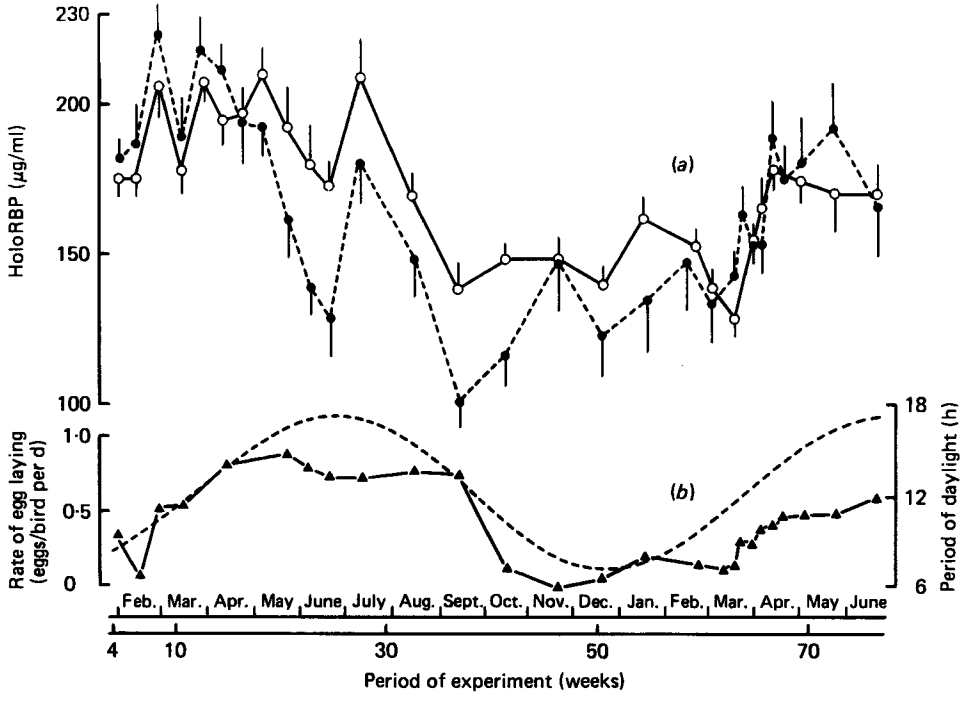

Fig. 3. Expt 3. (*a*) Annual changes in concentration (μg/ml) of plasma retinol-binding protein (holoRBP) in male (○—○) and female (●---●) groups of Japanese quail (*Coturnix coturnix japonica*) maintained under artificial light photoperiods corresponding to daylight occurring at 56° N latitude from February 1977 to June 1978. Points represent mean values with their standard errors represented by vertical bars. (*b*) The rate of egg-laying (no. of eggs/bird per d) by the female group (F_3) (▲—▲) in relation to the seasonal light–dark photoperiod (-----).

a range of interval mean values from 80 μg/ml to 113 μg/ml. Afterwards the concentration rose through the November–January period to maximal or high levels in the breeding period from March–May, with interval mean values from 164 to 185 μg/ml in the second cycle for F_2 and F_3. This concentration range was slightly lower than the corresponding one for F_1 and F_3 birds in their first cycle (184–247 μg/ml). In Expt 2 (Fig. 2) the highest mean concentration of holoRBP (240 μg/ml) was actually recorded for F_2 in January in the course of their second seasonal cycle but the average concentrations remained high at about 185 μg/ml up to May before significantly declining again. The F_3 birds under artificial light did not show maximal holoRBP concentration (190 μg/ml) until the April–May period in their second cycle. In this group and in F_1 the increase through November–January was much more gradual than in F_2.

Thus the major trend in plasma holoRBP in females is for the concentration to be high from February to May and low from August to October. In birds under natural lighting there was at least a 2-fold change in mean concentration between these periods. The elevation in concentration in group F_3 under artificial light in the spring was somewhat smaller at 1·5–1·7 times the late summer value.

Examination of the results for male birds shows that the over-all changes in holoRBP concentration were qualitatively similar to those found in females but quantitatively they were less marked in that the spring : autumn value in mean concentration was only 1·2 to 1·3. This lower value arises mainly from the fact that male plasma holoRBP concentration did not decline so much in the summer and autumn compared to that of the females.

Table 2. *Changes in mean plasma holoRBP concentrations (μg/ml) in groups of male (M) and female (F) Japanese quail between the successive periods shown in the annual cycle*

Expt	Group	No.	Jan.	Feb.–Mar.	Apr.–May	June–July	Aug.–Oct.	Nov.–Jan.	Feb.–Mar.	Apr.–May	June
			1976					1977			
1	M_1	4	123 } 116	200 } 206	218 } 233	156 } 150	163 } 126	158 } 145	—	—	—
	F_1	4	109 }	211 }	247 }	144 }	90 }	132 }			
	Change			77 } +90*****	18 } +27***	−62 } −83****	+7 } −24 NS	−5 } +19 NS			
			1976					1977			
2	M_2	5	—	—	181	165	174	191	172	213	201
	Change					−24*	+9 NS	+17 NS	−19 NS	+41**	−12 NS
	F_2	5	—	—	198	142	80	207	188	185	129
	Change					−56**	−62*	+127*****	−19 NS	−3 NS	−56****
			1977					1978			
3	M_3	9	—	184	200	187	153	149	139	170	167
	Change				+16 NS	−13*	−34*	−4 NS	−10****	+31*****	−3 NS
	F_3	7	—	187	184	140	113	124	131	164	156
	Change				−3 NS	−44***	−27****	+11 NS	+7 NS	+33**	−8 NS

NS, not significant. *$P < 0.05$, **$P < 0.025$, ***$P < 0.01$, ****$P < 0.005$, *****$P < 0.001$.

There also appears, however, to be a slight difference between the plasma values of the two sexes in the early spring, the concentration in females tending to be somewhat higher than that in the males in March and April in Expt 1 and 3 where first cycles were followed. In Expt 2 the concentration in the F_2 group in the second cycle also exceeded that of the M_2 group a little earlier in January and February. Consequently, the amplitude of the swing in plasma holoRBP concentration in females over the annual cycle was slightly greater than in males.

In addition to the above annual cyclic changes, a sharp but significant short term dip (about 15–20 %) in holoRBP concentration occurred after the major peak values in March in the first cycles of the smaller combined male and female groups in Expt 1 ($P < 0.01$) and in the individual groups M_3 ($P < 0.001$) and F_3 ($P < 0.02$) of Expt 3 before the concentration rose to the more sustained levels over April and May. Another characteristic feature of the cyclic changes is that the average concentration of holoRBP begins to decline after the April–May period as indicated in Table 2. The change is quite definite in the female groups but much less marked though just significant ($P < 0.05$) in the male groups, even in M_3 in spite of the unusually high single mean value recorded for mid-July.

Egg laying in all females occurred over the period April–October. The numbers were not recorded for F_1 and F_2 and none were laid beyond October. The total number of eggs laid by F_3 birds over the intervals between each plasma test were recorded and the average number/bird per d plotted in Fig. 3(*b*) along with the number of hours lighting the birds received during the experiment. From this it can be seen that the egg-laying rate was consistently high during the period April–September when the photoperiod was greater than 12 h and lowest in October–December.

DISCUSSION

The results confirm previous observations in sheep (Glover *et al.* 1976) in that plasma holoRBP concentration increases in association with the sexual development of the birds. Maximal values in Japanese quail are attained in the spring when the daylength increases, whereas it is autumn with decreasing daylength for sheep. Again, cyclic changes were observed in both sexes, but the minimal values for female birds fell considerably below those for males in late summer. In their pattern of changes there appear to be at least two major peaks in plasma holoRBP concentration during the sexually active period, as with that for sheep, although there are several minor fluctuations in concentration throughout the annual cycle. The minor changes arise partly at least from the fact that the stimuli within individual birds in each group would not necessarily be in perfect synchrony, and partly in Expts 1 and 2 from interassay errors of the successive analyses. However, the 2- to 3-fold increase in plasma holoRBP in females and 1·5-fold increase in males above the minimum levels during February–May just prior and at their breeding period, when changes in pituitary and other hormones take place, indicate hormonal involvement in the control of RBP biosynthesis and secretion from the liver. It has been shown in children that the plasma concentration of total RBP increases with the onset of puberty (Peterson *et al.* 1974). This has also been confirmed in experiments using the polyoestrous rat where similar minor fluctuations in plasma holoRBP occur during early development but major increases in holoprotein occur at puberty coincidental with increasing gonad weight (Kershaw, 1978).

The sharp 15–20 % reduction in holoRBP after the major peaks in March during the first cyclic increase for birds in Expts 1 and 3 is much greater than the experimental errors of the assay. This sudden decline in concentration is of relatively short duration since it is represented by determinations at only one time period in each experiment. Clearly this stage of the seasonal cycle needs to be examined in greater detail, but the significant drop

in concentration at that period may be partly related to a sharp increase in utilization of retinol. The elevated plasma level represents the net difference between increased synthesis and release of holoRBP from the liver and its utilization by the body tissues. The requirements of most of the latter, however, would only increase slightly commensurate with the general growth of the bird, but the gonads growing logarithmically over this period will presumably cause a marked increase in consumption of retinol to meet the needs of the increasing quantity of gonadal tissues as well as supplying the specific requirement for the proper differentiation of some of them.

Males. In male birds for example, testicular weight has been observed to increase from 10 to 3000 mg during their rapid development from mid-March to the end of April, but the greater portion of this growth occurs within approximately 2 weeks (Farner & Follett, 1966; Follett & Maung, 1978). Increased utilization of retinol by the developing organ could possibly cause a temporary lowering in plasma holoRBP. Once the organ has reached its full functional size its demand for retinol will be reduced to that necessary for the maintenance only of the tissue so the plasma concentration will rise to the second maximum provided the higher output of the protein by the liver is maintained.

The elevated concentration of holoRBP after the initial surge in March is held for approximately 2 months over April and May, by which time the birds have reached maturity as judged by the size of the cloacal gland. This is approximately the time period when Follett & Maung (1978) found the testes to be fully grown in groups of Japanese quail maintained under similar natural lighting conditions over a seasonal cycle. By the end of May and early June holoRBP concentration had fallen well below the average spring maximum in all experiments as can be seen from Figs 2 and 3. This suggests that either the utilization of retinol from plasma holoRBP must be increasing more rapidly than its secretion by the liver or that the secretion itself is beginning to decrease. The latter explanation is preferred since the amount of retinol required to maintain the developed organ should be less than that required for its growth and maintenance. Thus, once the birds have matured and tissue development has ceased, the retinol supply from the liver appears to become restricted. An appreciable decline in testicular weight, however, does not occur until late July or August when plasma holoRBP has settled down to 150–170 μg/ml, the minimal range for the seasonal cycle in male birds.

When the major variations in holoRBP are compared with the pattern of growth and regression of testicular tissue in the males as observed by Follett & Maung (1978), it seems that the initial surge in holoRBP occurred coincidentally with the initiation of the growth of the tissue by the hormones of the hypothalamo-pituitary axis responding to increasing daylength. The first prolonged reduction in holoRBP, however, was evident in May–June in all experiments (see Table 2) before significant regression of the testes normally occurs in late July–August (Follett & Maung, 1978) and also at a time period when the daylength is still increasing or at least greater than 15 h. Even in Expt 3 under artificial light where a short-term further peak in holoRBP was observed in July, the subsequent decrease in concentration occurred when the light period is much longer than the dark one. This suggests that the general reduction in plasma holoRBP concentration in mid- to late summer is probably brought about by a control mechanism different from the initiating photoperiodic one. Just as in sheep where plasma holoRBP increased with shortening daylength so too the decreasing phase took place when the days were even shorter (Glover *et al.* 1976).

Whatever the control system involved, it is quite likely that the rate of synthesis of a liver secretory protein such as RBP with rapid turnover will be seen to respond more quickly to inhibition than will the breakdown of the more complex gonadal tissue, so a lag in the regression of the latter after holoRBP is not surprising.

Females. The annual pattern of variation in plasma holoRBP for females was similar to that of the males but the amplitude of the cyclic changes was slightly greater in that the concentration tended to be higher than that in the male groups in March and April and lower in September and October. This difference probably reflects the need for females initially to release extra retinol from the liver for transfer to the eggs but later the additional demand on the supply of retinol from plasma holoRBP by egg laying continues beyond the period when the controlled output of the holo protein declines again. Some RBP is itself transferred to the egg in addition to retinol (Heller, 1976; Heaf & Glover, 1979).

Although some retinol is found in egg yolk in the form of holoRBP, it does not imply that all the vitamin is transferred to the yolk in this form. The maximum egg-laying period occurred in May but was high from April to September (see Fig. 3*b*) when each bird produced approximately one egg/d containing approximately 30 μg retinol which is more than the amount contained in the plasma pool at any one time. Thus the synthesis and secretion of holoRBP by the liver in the female over this period must be increased considerably over that needed in the sexually quiescent period.

Once egg production had ceased, however, the drain on retinol supply was reduced so that the plasma level of holoRBP returned during the November–January period to the range of mean values (140–190 μg/ml) found in males at this time. Since the mean concentration of holoRBP for male groups does not change significantly from August to December this could perhaps be regarded as the basal level for sexually quiescent birds.

In the second cycles of Expts 2 and 3 the patterns of change in mean values were different from and more variable than that for the first cycle. In Expt 3 the really sharp increase in holoRBP took place at the end of March in both M_3 and F_3 groups. In Expt 2 this was also true for M_2, but in F_2 the concentration was already elevated following a temporary sharp increase in January. There was also a small temporary but significant increase ($P < 0{\cdot}025$) in the M_3 group during January. Inspection of the changes in individual birds, however, indicates that the degree of synchrony in holoRBP concentration at this early part of the year is poorer than during the first cycle, indicating that other factors in addition to daylength change are affecting the secretion of holoRBP during this period. Although no satisfactory explanation can be offered for this, it is clear that in the spring period, both cycles show maximal or elevated plasma holoRBP. This has also been confirmed by the determination of total immunoreactive retinol binding protein concentration (Heaf & Glover, 1979). The photoperiod at this time has been shown by Follett (1976) to provide the minimum daylight necessary to trigger the development of the gonads and produce marked changes in the secretion of gonadotrophins.

Hormonal relationships. The nature of the hormone linking the changes in the hypothalamo-pituitary-gonadal axis with the secretion of RBP has still to be elucidated. Since previous experiments in sheep showed that the cyclic changes in RBP occurred in wethers as well as ewes (Glover *et al.* 1976) the gonadal steroid hormones are not considered likely to be involved either in stimulating the increased synthesis of RBP by the liver, or in the feedback inhibition of holoRBP, but adrenal steroids cannot be ruled out.

It is, however, well established that the secretion of several proteins from the liver can be stimulated by steroid hormones, for example oestrogen is capable of stimulating vitellogenin secretion for the deposition of lipovitellin and phosphovitin in eggs (Wallace & Jared, 1969; Tata, 1976). Some work has also been done on the action of this hormone on plasma retinol. Chapman *et al.* (1949) reported that the administration of increasing doses of oestradiol benzoate along with a smaller amount of testosterone to immature pullets caused plasma retinol levels to increase in relation to the dose. Yet injection of 6-week-old rabbits with oestradiol alone did not affect plasma retinol concentration

(Williamson, 1947). More recently it has been found that women taking oral contraceptives have plasma vitamin A levels about 50% above normal (Gal *et al.* 1971), but it required very high doses (fifty times normal in proportion) to be administered to female rats to bring about a significant increase in plasma retinol bound to RBP (Supopark & Olson, 1975). The changes observed in seasonal breeding animals, however, probably arise in a different manner from the above more pharmacological effects, since both sexes are involved.

Experiments are in progress to examine the effects of various hormones on RBP secretion and information on the temporal sequence of changes in the luteinizing hormone and in total RBP are reported elsewhere (Heaf & Glover, 1979).

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