Vol. 137, No. 2 October, 1969

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

CONTINUOUS AND RHYTHMIC REPRODUCTIVE CYCLE OBSERVED IN *PERIPLANETA AMERICANA* (L.)

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The secretion of vitellogenic proteins (vitellogenins) by the fat body into the blood and their sequestration by the oocytes have been found in the reproductive cycles of *Periplaneta americana* (Mills, Greenslade and Couch, 1966; Adiyodi and Nayar, 1967; Nielsen and Mills, 1968), *Leucophaea maderae* (Engelmann and Penney, 1966) and *Nauphoeta cinerea* (Adiyodi, 1967). Because these cycles differ in many respects, including their duration, there are basic differences in the schedule of vitellogenin secretion and yolk formation.

The purpose of this communication is to elucidate the reproductive functions of blood proteins in *Periplaneta americana,* including its two vitellogenins, by correlating their fluctuations with cyclic changes in the rate of yolk deposition and with other phases of reproduction. Precise information on these relationships from a number of species with varying cycle characteristics should provide better insight into the cellular processes and the control mechanisms which govern reproduction.

Methods and Materials

Colonies of *Periplaneta americana* were fed Purina laboratory chow and water, and maintained at 26° C, 70% relative humidity, with a 12:12 hour photoperiod in a constant temperature incubator. Adult females were staged according to the time after the ootheca begins to form (BF); the time of ootheca formation was designated as day 1.

Staged females were selected every 24 hours after ootheca formation. Samples of blood were collected according to the method of Mills *et al.* (1966), and the ovaries were dissected out in Ringers solution (van Asparen and van Esch, 1956). The length and width of the basal and penultimate oocytes were measured and the volume was calculated using the formula for a prolate spheroid,

$$V = (W/2)^2(L/2)(4/3\,\pi).$$

Yolk proteins were obtained either by homogenizing oocytes removed from the ovaries or by collecting the contents of newly formed oothecae. Ten or twenty oocytes were homogenized in 0.4 *M* NaCl buffered at pH 7.2 and the mixture was centrifuged at 10,000 *g* for 30 minutes in the cold. A lipid cap rested on the clear supernatant and a sediment of membrane and ovarian sheath formed a pellet at the bottom of the tube. When oocytes were broken in 0.4 *M* NaCl and

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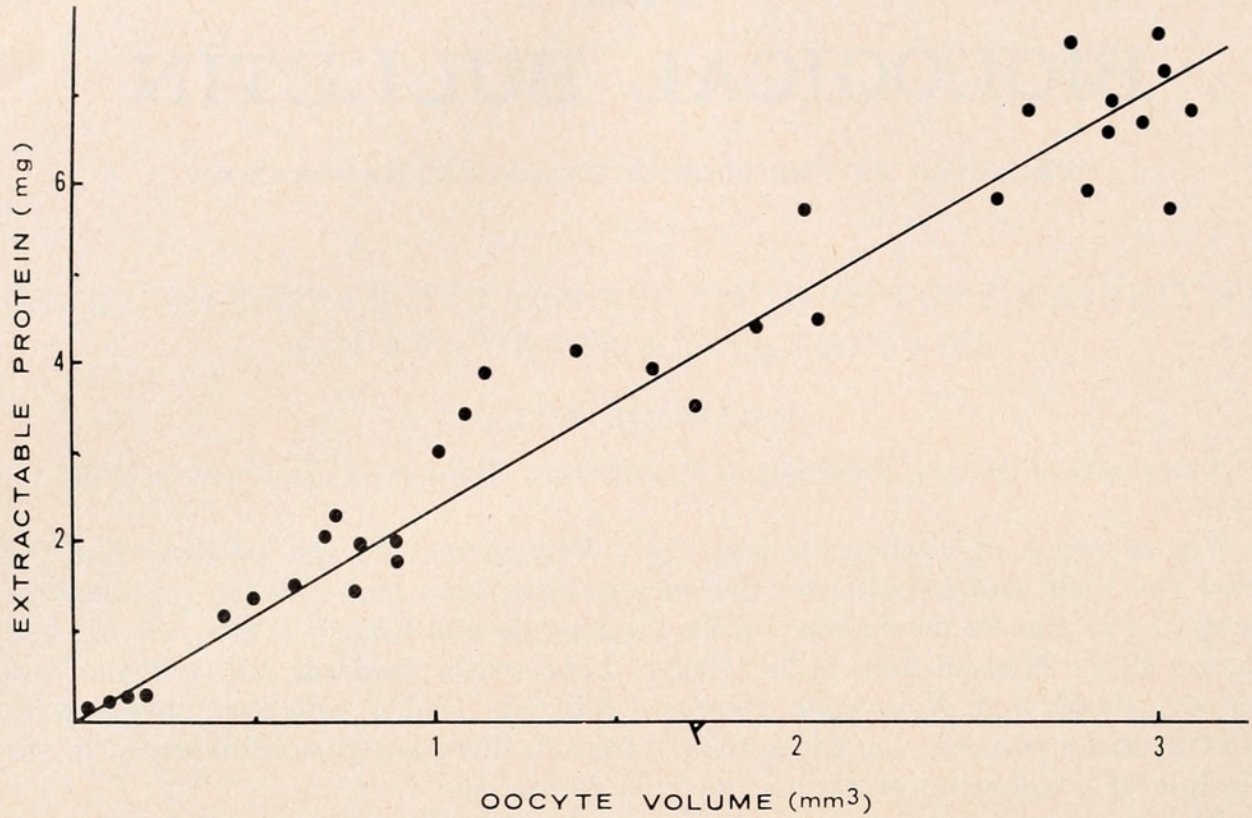

FIGURE 1. Protein content compared with volume in vitellogenic oocytes. Each point is the average of 10 oocytes.

the behavior of yolk fluid observed under low power microscopy, the viscous protein fluid released from broken yolk spheres became soluble; when broken in 0.15 *M* NaCl, precipitation was noted. Thus the necessity of high ionic strength for oocyte protein solubility, reported by Dejmal and Brookes (1968) for *Leucophaea maderae,* was also true for the oocyte proteins of *Periplaneta.* Yolk fluid squeezed from newly formed oothecae, however, differed in this regard. The yolk fluid was centrifuged as above and the protein fraction was removed and a serial dilution was performed in 0.15 *M* NaCl. Oocyte proteins failed to precipitate when diluted to 5%; below this critical point of dilution, the proteins were precipitated.

Cockroach blood and the supernatant resulting from the centrifugation of homogenized oocytes were measured for total protein concentration using the microbiuret method of Itzhaki and Gill (1964). Vitellogenin concentration was measured by the quantitative immunodiffusion technique (Oudin, 1948) utilizing an antiserum prepared against the yolk protein fraction from newly formed oothecae (Bell, 1969a). The antiserum, solidified with agar in glass tubes, was overlayered with blood or yolk fluid and the rates of migration of the zones of precipitation were recorded. The concentration of the two vitellogenins in female blood, relative to their concentrations in yolk fluid, was calculated by referring to a standard curve of the log of yolk fluid antigen concentration plotted against the rate of migration.

Blood volume determinations were made using the C^{14}-inulin method described by Wharton, Wharton and Lola (1965).

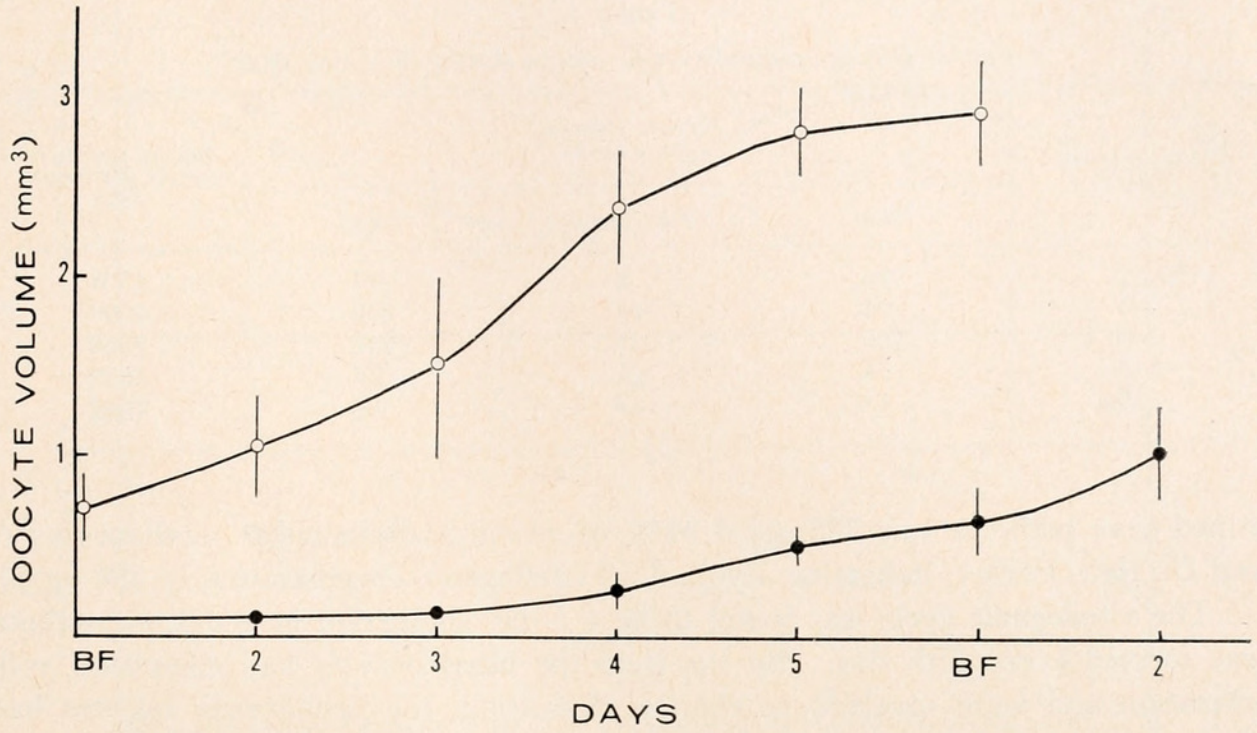

FIGURE 2. Oocyte volume during the 5-day cycle. Each point is the average of 25 to 50 females. Vertical lines are standard deviations [basal oocytes ○, penultimate oocytes ●].

RESULTS

Changes in oocyte volume and protein content during the vitellogenic cycle

The volume of the oocytes increased proportionately with the increase in extractable yolk protein (Fig. 1). The total protein concentration of yolk fluid ob-

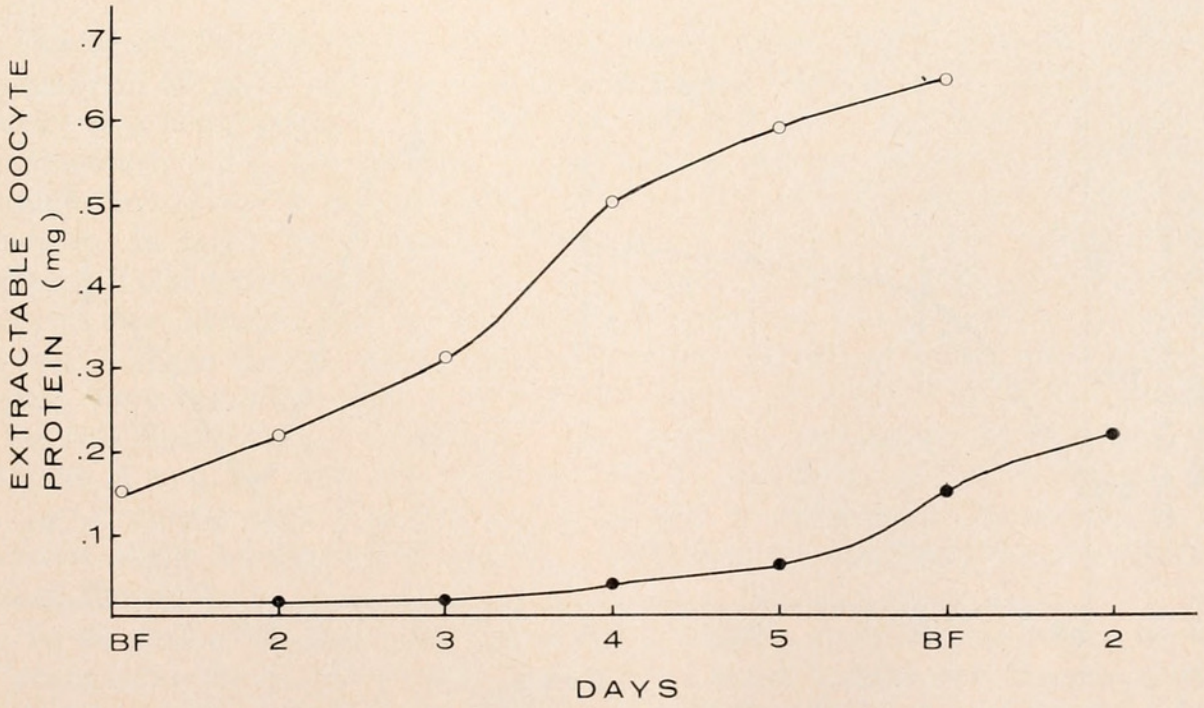

FIGURE 3. Oocyte protein content during the 5-day cycle. Each point is the average of 25 to 50 females [basal oocytes ○, penultimate oocytes ●].

TABLE I

Rate of protein accumulation by oocytes during the 5-day cycle

Time interval (days)	Protein increase (μg/oocyte)			Oocyte protein increase per female (μg)
	Basal	Penultimate	Total	
BF–2	86	8	94	1504
2–3	94	12	106	1690
3–4	160	25	185	2960
4–5	74	21	95	1520
5–BF	43	43	86	1344

tained from oothecae was 225 μg/μl, 88% of which is attributed to vitellogenins A and B (Bell, 1969a), indicating a yolk fluid vitellogenin concentration of 198 μg/μl.

The vitellogenic cycle was found to be a 5-day succession in which an ootheca was formed every fifth day. By the time the basal oocytes had completed yolk formation and were involved in chorion deposition, the penultimate oocytes had reached an advanced stage in yolk formation and continued during and after the basal oocytes were ovulated; thus two oocytes in each ovariole were supporting yolk deposition simultaneously (Roth, 1968). This relationship was observed in both the volume (Fig. 2) and the protein content (Fig. 3) of the oocytes. The continuum is not regular, however, as the amount of protein accumulated by the combined effort of the basal and penultimate oocytes (Table I) differed from one stage in the cycle to another. There was a peak of yolk formation between days 3 and 4, whereas at either end of the cycle the rate was about half that at the peak.

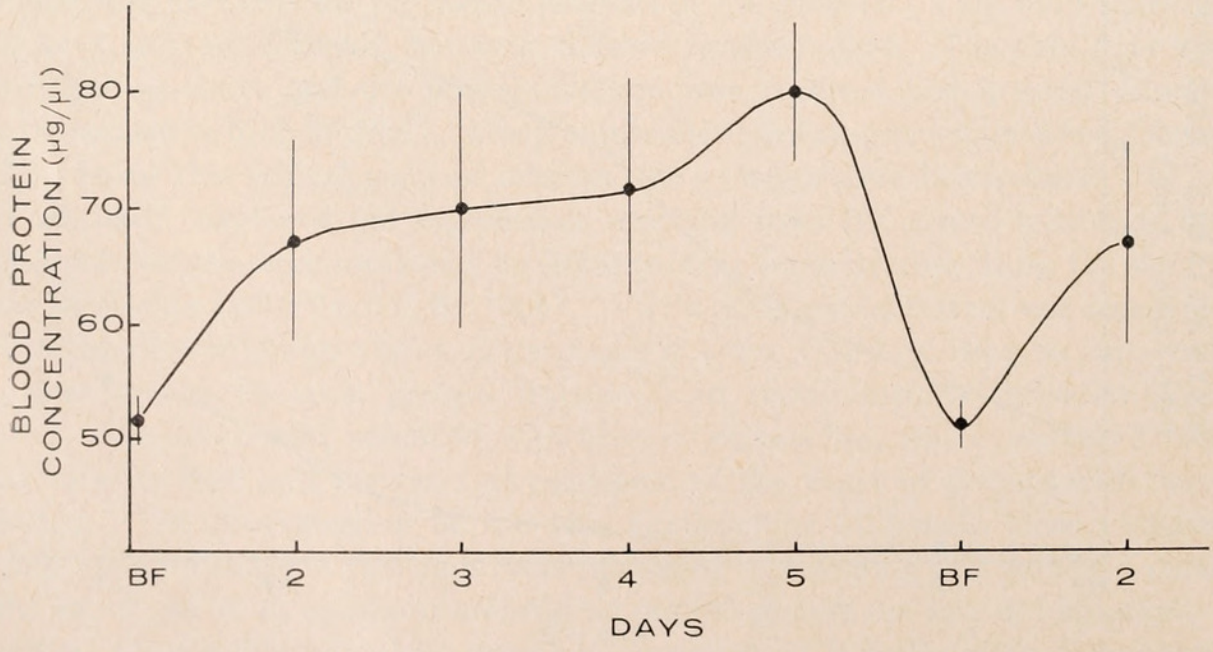

FIGURE 4. Total blood protein concentration during the 5-day cycle. Each point is the average of 20 females. Vertical lines are standard deviations.

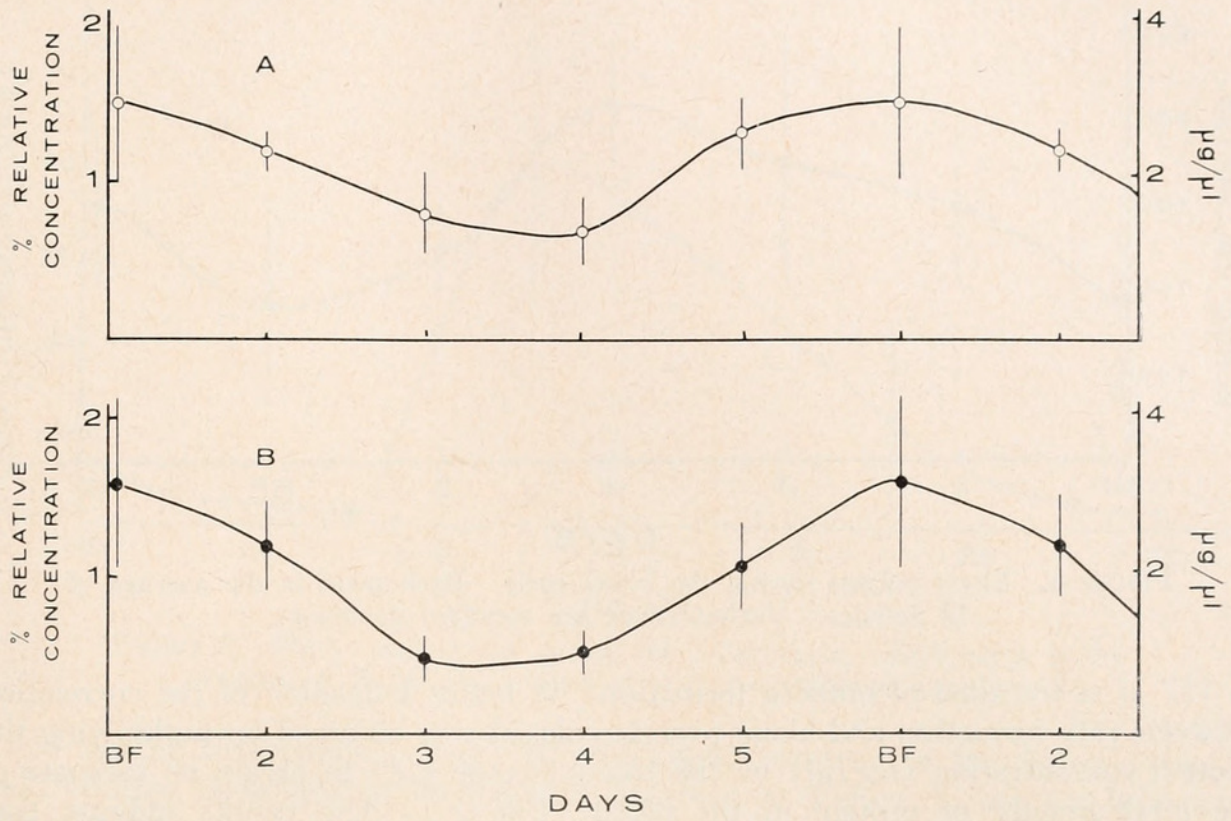

FIGURE 5. Blood vitellogenin concentration during the 5-day cycle. Each point is the average of 20 females. Vertical lines are standard deviations. Per cent concentration is relative to the concentration in the yolk fluid standard, left; estimated protein concentration in μg/μl, right; vitellogenin A ○, vitellogenin B ●.

Changes in blood protein and vitellogenin concentration during the vitellogenic cycle

During the 5-day cycle the total blood protein concentration (Fig. 4) increased gradually to 80 μg/μl on day 5 and then decreased abruptly to 52 μg/μl while the ootheca was being formed. The levels of vitellogenins A and B, which oscillated synchronously (Fig. 5), reached a peak during ootheca formation and declined through days 3 and 4 of the cycle. Vitellogenin concentration in the blood was therefore, as might be expected, lowest when the rate of yolk formation was highest. Total blood protein, on the other hand, while undergoing a 5-day cycle, dropped most precipitously on days 5 to BF when the rate of yolk formation was minimal. When the per cent vitellogenin figures (resulting from Oudin measurements) were converted into μg/μl by referring to the total protein concentration of the yolk fluid standard (225 μg/μl), the combined vitellogenin concentration (of antigens A and B) was equivalent to only 6.5 μg/μl at ootheca formation and 2.0 μg/μl during days 3 and 4. The changes in vitellogenin concentration are therefore slight compared with the 30 μg oscillation in total blood protein observed during the cycle.

To determine whether the fluctuations in protein concentration might be due to changes in the blood volume, the average blood volume for females during each stage of the cycle was measured. The results, depicted in Figure 6, show that the blood volume gradually increased to 207 μl at day 4 and then dropped

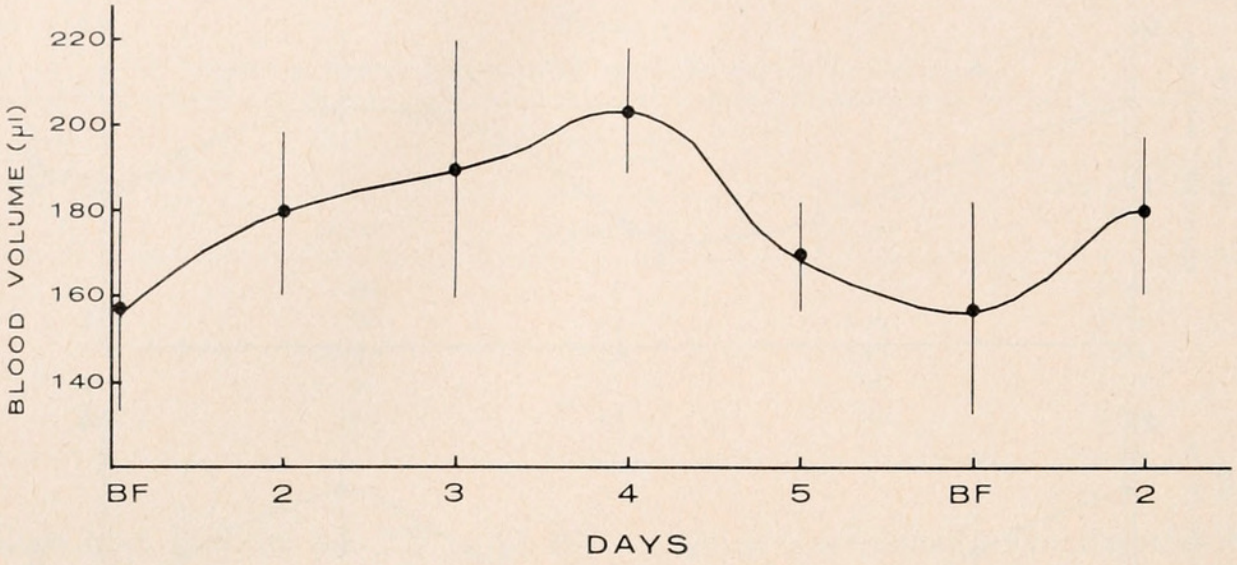

FIGURE 6. Blood volume during the 5-day cycle. Each point is the average of 10 females. Vertical lines are standard deviations.

to 157 μl at the time of ootheca formation. A better indication of the correlation between yolk formation and blood protein content was obtained by multiplying the protein concentration (mg/μl) by the blood volume (μl) to obtain an estimate of the total amount of protein in the blood (Fig. 7). The results indicate that changes in blood volume do not explain the blood protein concentration changes shown in Figure 4, but, on the contrary, amplify the drop in protein correlated with ootheca formation.

The changes in the total blood protein must be due to changes in the relative concentration of non-vitellogenic blood proteins which do not have homologous antibodies to the yolk proteins in the antiserum utilized in this study. That non-vitellogenic blood proteins undergo alterations in relative concentration in female *Periplaneta* is also suggested by electrophoretic analyses of blood proteins during the vitellogenic cycle (Nielsen and Mills, 1968; Adiyodi and Nayar, 1967).

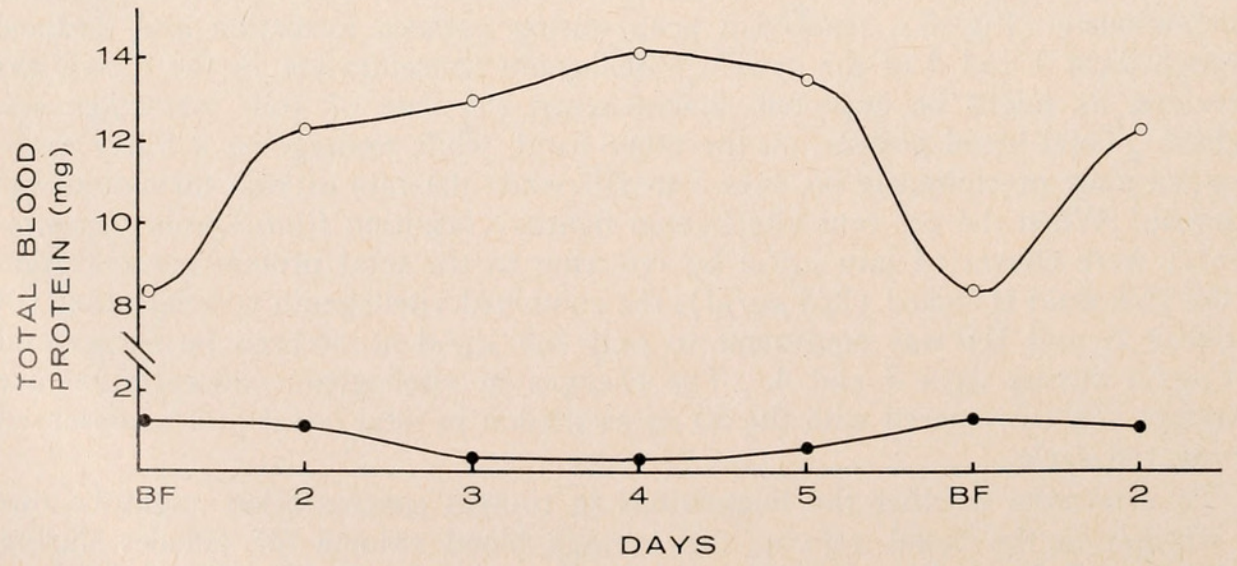

FIGURE 7. Estimation of blood protein content during the 5-day cycle. Total protein is designated as ○, vitellogenin A and B combined as ●.

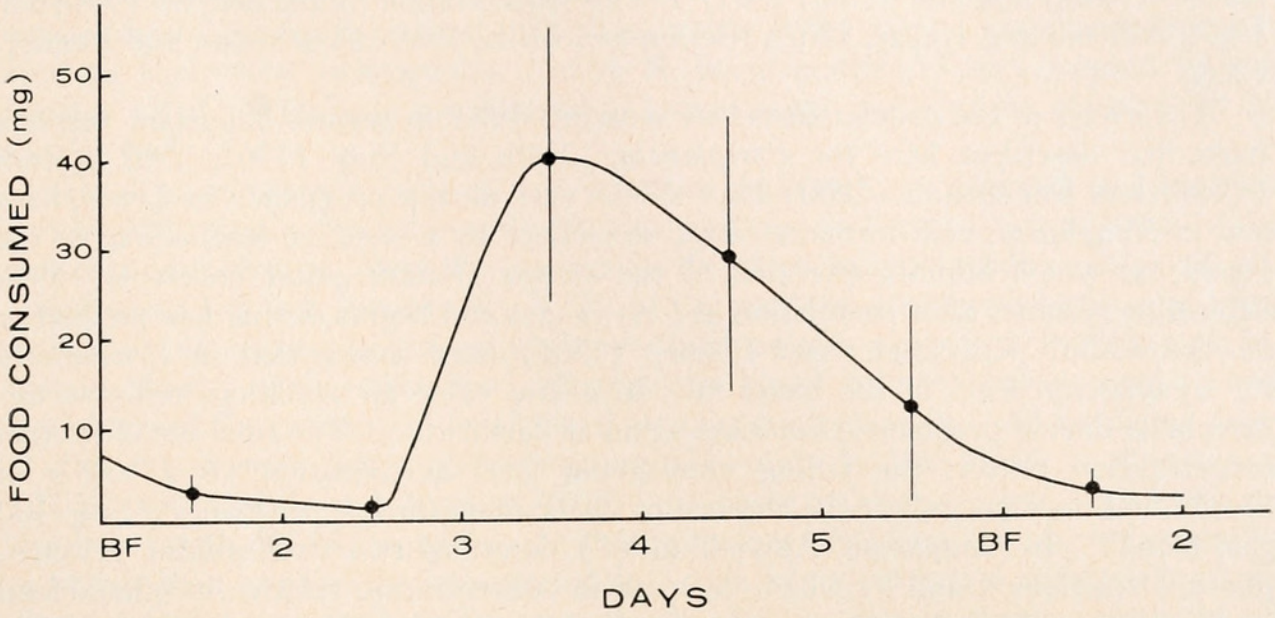

FIGURE 8. Food consumption during the 5-day cycle. Each point is the average of 30 females. Vertical lines are standard deviations.

A similar treatment of the total vitellogenin content of the blood (Fig. 7, solid circles) dampens the fluctuations observed in Figure 5, but the effects of yolk deposition remain apparent. Thus the vitellogenin content of the blood is lowest on days 3 and 4, during the period when yolk deposition is most rapid (Table I), and substantially higher during periods of reduced yolk deposition. That there should be such an effect is not surprising, considering the fact that 9 mg of vitellogenins A and B combined are incorporated into the oocytes during one ovarian cycle, whereas the blood contains at any one time no more than 1.0 mg.

A final indication of cyclicity correlated with ovarian development was observed in the feeding cycle of reproductive females (Fig. 8). The amount of food consumed by vitellogenic females was estimated by weighing the food pellets daily for 30 animals over a 60 day period. There is much variation between individuals, but a hiatus in feeding is evident at the time of ootheca formation, whereas a peak in feeding is observed during the nights of stages 3 and 4. The correlation of food intake with the 5-day cycle might have been expected to provide an explanation for the rise in blood protein content during the first two days after ootheca formation; feeding however, is not initiated until the rise in blood protein is nearly completed. This result necessitates the prediction that there is a cycle in intracellular protein storage in tissues such as the fat body (Dr. Barbara Stay, Department of Zoology, University of Iowa in preparation).

DISCUSSION

There is a wide range of reproductive diversity in cockroaches, extending from oviparity (*Periplaneta americana*), and ovoviviparity (*Leucophaea maderae, Nauphoeta cinerea*) to a resemblance of viviparity (*Diploptera punctata*) (Roth and Willis, 1958). This closely related group of insects thus offers various types of reproductive cycles for study. All cycles which have been adequately investigated seem, nevertheless, to entail as one component the secretion of vitellogenin by the

fat body (Pan, Bell and Telfer, 1969) and its sequestration by the oocytes (Adiyodi, 1967; Adiyodi and Nayar, 1967; Nielsen and Mills, 1968; Engelmann and Penney, 1966).

The cycles of the ovoviviparous cockroaches differ in several important respects from that described here for *Periplaneta.* Roth and Stay (1961, 1962), Roth (1964) and Engelmann (1960) have shown that during pregnancy in *Leucophaea* and in *Nauphoeta,* yolk formation may be blocked by a negative feedback from the brood sac which inhibits secretion of the *corpus allatum* gland hormone. Yolk deposition resumes after parturition in *Leucophaea* and begins during late pregnancy in *Nauphoeta.* Engelmann and Penney (1966) have shown that in *Leucophaea* the vitellogenin level in the blood falls to a low value at ovulation, remains undetectable during pregnancy, but rises again at parturition. The total blood protein concentration reflects the falling vitellogenin level at ovulation (6 g/100 g of blood), but does not reflect the absence of vitellogenin during pregnancy (10 g/100 g of blood). In *Nauphoeta,* Adiyodi (1967) described two vitellogenins (electrophoretic fractions 4 and 5) which decrease in concentration relative to other blood proteins during yolk formation, comprise less than 10% of the total blood protein complement at ovulation, and then increase gradually, especially during middle and late pregnancy, to a 20 and 45% of the total blood protein complement.

Two interesting points emerge from these studies. First, during pregnancy, inhibition of the *corpus allatum,* promoted apparently through the proprioception of egg incubation (Roth and Stay, 1961) or humoral ovarian factors (Engelmann, 1964), recedes gradually toward the end of pregnancy (in *Nauphoeta* and *Diploptera*) allowing juvenile hormone secretion. One of the functions of juvenile hormone is to promote the synthesis of vitellogenin (Coles, 1964; Minks, 1967; Thomas and Nation, 1966; Engelmann and Penney, 1966; Bell 1969b), and this presumably accounts for the increase in blood vitellogenin and the initiation of yolk formation during late pregnancy in *Nauphoeta.* In *Leucophaea,* yolk deposition in the next batch of oocytes fails to occur until parturition, and this correlates with the absence of vitellogenin in the blood of pregnant females. Secondly, in the two ovoviviparous species studied, the changes in total blood protein concentration reflect the changes in vitellogenin levels to a different degree. In *Leucophaea* the total blood protein level appears not to correlate with the vitellogenin level, while in Nauphoeta the vitellogenins occupy a large percentage of the total blood protein and seem to be responsible for changes in the total blood protein levels. Because there is a close correlation between yolk formation and depletion of total blood protein resources in *Pyrrhocoris apterus* (Sláma, 1964) and in *Schistocerca gregaria* (Hill, 1962) it is probable that the vitellogenins occupy a large part of the blood protein in these species also.

Reproduction in *Periplaneta* differs from that in ovoviviparous cockroaches in that the eggs are not incubated internally, and the ovarioles contain two oocytes (basal and penultimate) in zone V which are simultaneously forming yolk (Bonhag, 1959; Roth, 1968). Moreover, vitellogenesis is continuous in the American cockroach, although cyclic variability is observed in the rate of this process (Table I). In contrast to the accumulation and depletion of vitellogenins, as in *Leucophaea* and *Nauphoeta,* the vitellogenic proteins of *Periplaneta* do not accumulate in the blood and comprise only about 4% of the total blood protein (Bell, 1969a). Nor are the blood vitellogenins significantly depleted during yolk formation; instead,

their concentrations in the blood remain relatively constant, with at most a 50% concentration change correlated with fluctuations in the rate of yolk formation. It appears likely that vitellogenins A and B, being selectively incorporated by the oocytes (Bell, 1969a) are sequestered at a rate equal to their rate of secretion by the fat body.

Total blood protein levels in the American cockroach, as depicted in this paper and by Mills *et al.* (1966), are not obviously related to the vitellogenic cycle. The concentration changes observed are nearly the same in both studies, although different assay methods were used (R. R. Mills, personal communication), and must eventually be related to the schedule of blood protein synthesis, feeding activity, and to blood protein utilization. Changes in blood volume appear not to cause the observed fluctuations in total blood protein concentration. As predicted from studies on diuretic and antidiuretic hormone titers in the blood of *Periplaneta* (Mills, 1967; Mills and Nielsen, 1967), the blood volume reaches a peak on the third or fourth day of the cycle. At the same time the blood protein concentration is higher than at other periods during the cycle; thus correction for blood volume changes accentuates, rather than compensates, for the protein changes in the blood.

The abrupt decrease in blood proteins prior to and during ootheca formation, which was not observed by gravimetric methods (Pratt, 1967), may be attributed to one or several possible causes. Fat body protein synthesis on the fifth day of the cycle is at least 40% lower than on other days in the cycle, and thus the low blood protein may represent in part a hiatus of protein secretion (Pan *et al.*, 1969: Bell, 1969a). Another possible reason for the drop in blood protein may be the utilization of blood proteins by the colleterial glands for the production of the ootheca, a proteinaceous structure which weighs 20 mg. Electrophoretic protein fractions common to the blood and to the colleterial glands have been demonstrated in *Periplaneta* (Adiyodi and Nayar, 1966) and in *Nauphoeta* (Adiyodi, 1968). The structural proteins of the ootheca, however, do not have counterparts in the blood, and Adiyodi concludes, in agreement with earlier work by Pryor (1940), that the left colleterial gland sequesters and hydrolyzes specific blood proteins to obtain amino acids for the synthesis of structural ootheca proteins. In analogous work, Brunet (1952) has reported that the composition of the cuticle and ootheca of *Periplaneta* are biochemically similar, and at least one blood protein disappears from the blood during the molt in several insects (Steinhauer and Stephen, 1959; McCormick and Scott, 1966; Chen and Levenbook, 1966). Sequestration of specific blood proteins by the colleterial glands, as in other insect tissues (Locke and Collins, 1967; Laufer and Nakase, 1965), might explain the decrease in *Periplaneta* blood proteins during ootheca formation.

The author is grateful to Dr. William H. Telfer for his invaluable advice during the course of this study. This work was supported by research grants from NSF (GB-4463) and NIH (5-TO1-GM-00849-07).

Summary

1. The 5-day reproductive cycle of *Periplaneta* is continuous with an ootheca being formed every fifth day. The rate of yolk formation is lowest during ootheca formation (day 1) and reaches a peak during days 3 and 4.

2. Vitellogenic blood protein levels fluctuate between 1 and 6 μg/μl and their concentrations correlate with the rate of yolk formation. The two vitellogenins oscillate synchronously during the cycle.

3. The total blood protein concentration increases to 80 μg/μl on day 5, decreases abruptly to 52 μg/μl when ootheca formation is initiated, and increases gradually to the concentration peak on day 5. The changes in total blood protein concentration are not obviously correlated with the vitellogenic cycle.

4. Changes in blood volume occur during the cycle, and these changes amplify rather than diminish the blood protein fluctuations.

5. A hiatus in food consumption is observed during ootheca formation; feeding is resumed after the third day following oviposition.

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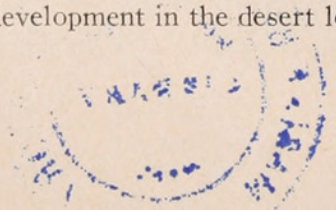

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