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VITELLOGENESIS IN A PRIMITIVE TERMITE, *ZOOTERMOPSIS ANGUSTICOLLIS* (HAGEN) (HODOTERMITIDAE).

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The sources and controls of egg storage proteins, the vitellins, have been subject to increasing attention in the past decade. Vitellogenesis offers an attractive model system in which large amounts of specific proteins are produced in a relatively short period of time, often under the direction of gonadotrophic hormones. In non-mammalian vertebrates a serum precursor to vitellin, a vitellogenin, has been demonstrated to be synthesized in the liver under the direction of estrogen. No other extra-ovarian tissue has been seriously implicated as a major source of egg storage proteins prior to ovulation. Subsequent to ovulation the oviduct donates a number of proteins which surround the egg. However, γ-globulins are specifically taken up into developing avian oocytes in small amounts serving a presumably immunological-protective rather than a nutritive role (Roth, Cutting and Atlas, 1976). While endogenous synthesis of proteins presumably occurs in all oocytes, in non-mammalian vertebrates it does not amount to a large proportion of stored protein (current review, Wallace, 1978).

In the higher invertebrates the source of yolk proteins has not been found to be consistently extra-ovarian. In the octopus, vitellin seems to be primarily an endogenous product of the ovary (O'Dor and Wells, 1973, 1975). In various crustaceans, despite earlier evidence of extraovarian synthesis of vitellogenins, current reports are convincing that the ovary is the major source of vitellin (Lui, Sage and O'Connor, 1974; Lui and O'Connor, 1976). More extensive studies in insects have established the fat body as the major source of vitellin (current reviews: Engelmann, 1978; Hagedorn and Kunkel, 1978; Wyatt and Pan, 1978). However, despite the fat bodies' preeminence as primary suppliers of vitellin, an ovarian source of yolk protein synthesis has been firmly established for the giant silkworm and, more specifically, the source of this endogenous ovarian protein is the follicle cells (Anderson and Telfer, 1969; Bast and Telfer, 1976).

Our knowledge of the repoductive physiology of social insects is still in an infancy stage, primarily due to the small proportion of each colony which is actively reproductive. Where termites stand relative to other insects with regard to the vitellogenic process is still in doubt. Studies with physogastric queens of the termite *Macrotermes subhyalinus* (Rambur) (Termitidae), which are capable of laying up to 40,000 eggs per day, have been interpreted to suggest that the follicle cells of the ovarioles are responsible for the production of vitellogenins (Wyss-Huber and Lüscher, 1975). Despite the tentative nature of this later weak inference, the termite queens' ability to reproductively support the large monogynous societies of the higher termites is nonetheless unsurpassed among the eusocial insects, and indeed, among the Insecta and the unique social structure and development of termites in general, may have resulted in modifications or exag-

gerations in the production of vitellogenic proteins compared to other nonsocial insects. Our limited knowledge of the structure and especially the physiology of the termite reproductive system (Weesner, 1969) must be extended. By taking advantage of our current understanding of termite evolution (Emerson and Krishna, 1975) we can perhaps approach an understanding of the origins of the remarkable reproductive capacity of the higher termites. In that light, we have undertaken an investigation of the development of the neotenic reproductive of the primitive west coast damp-wood termite *Zootermopsis angusticollis* (Hagen) (Hodotermitidae). Various aspects of vitellogenesis in this species were studied. We report here the isolation and TEAE-cellulose purification of vitellins from eggs of neotenic reproductives and immunological comparison with vitellogenins from adult and neotenic reproductives. By measuring vitellogenin production relative to oocyte maturation, we offer tentative proof of extraovarian synthesis of vitellogenin. Initial molecular weight estimates of the vitellin subunits also characterize these termite vitellins as similar to the extraovarian vitellins of other insects.

Materials and Methods

Culturing of animals

Stocks of *Zootermopsis angusticollis* were obtained from the Del Monte Forest, Pacific Grove, California. Colonies were maintained in wood contained in 37.5 × 20.5 × 17.5 cm plastic boxes at ambient room temperature (25° C). Neotenic reproductives (also termed supplementary or secondary reproductives) were obtained in the following manner: larvae were collected from laboratory stock colonies and maintained in isolated groups, without a functional queen or king, in petri dishes containing wet filter paper and wood from the stock colony. These groups were kept in an incubator at 24° C and 24 hr light. Under these conditions competent larvae molted to neotenic (non-winged) secondary reproductives and began laying eggs 25 days (range 22 to 30 days) after the neotenic molt. For a discussion of the termite neotenic reproductive caste see Miller (1969). Adult (winged) primary reproductives were taken from incipent colonies which had been established in the laboratory.

Preparation of crude yolk sample

Eggs were collected daily from colonies headed by neotenic reproductives and stored at 4° C. Crude yolk was prepared by homogenizing eggs over ice in distilled water. An equal volume of 0.4 N NaCl saline (0.4 N NaCl, 0.02 N PO_4, 0.02% sodium azide, pH 7.5) was subsequently added with further homogenization. The homogenate was centrifuged at 14,000 × *g* for 15 min. at 4° C in an International B-20 refrigerated centrifuge and the supernatant, between the floating lipid layer and the pellet, was collected and stored at 4° C. This constituted the crude yolk sample.

Purification of vitellin from crude yolk

Crude yolk was purified on a TEAE-cellulose (Cellex T, Biorad) column after Pan and Wallace (1974). The column (0.5 × 5.0 cm) was equilibrated with 0.1

N starting buffer (0.1 N NaCl, 0.005 N Na_4EDTA, 0.0125 N Na_2PO_4, pH 6.5) and the crude yolk sample (from 18 mg of eggs) in its, 0.2N NaCl buffered saline diluted 1 : 1 with distilled water, was run onto the column. The sample was eluted from the column with starting buffer in steps of increasing NaCl concentration and, using immunochemical means of identification, the fractions containing the purified yolk (0.3–0.4 N NaCl) were pooled and vacuum dialyzed overnight in the cold against 0.15 N NaCl saline.

Immunological procedures

Antisera were obtained by immunizing two rabbits with purified yolk emulsified (1 : 1) in Freund's complete adjuvant. Blood was taken from the ear vein one week following a booster injection and the γ-globulins were prepared by precipitation with one-third saturated $(NH_4)_2SO_4$. Quantitative (rocket) immunoelectrophoresis was performed on glass slides covered with 0.8% agarose containing antisera following the methods of Axelson, Kroll and Weeke (1973) using a Tris-EDTA-Citrate buffer, pH 8.6. Hemolymph samples from termites were obtained by pricking the abdomen and collecting the blood in 1 μl glass capillary tubes. The blood was mixed with 20 μl 0.15 N NaCl saline and applied to wells (2 mm diameter, capacity approximately 3.6 μl) which had been precut in the agarose.

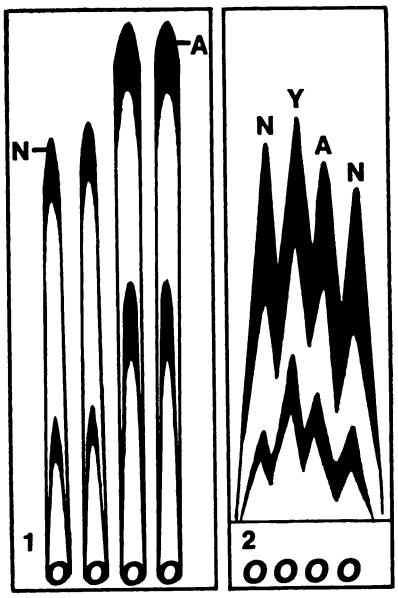


FIGURE 1. Schematic drawing of quantitative "rocket" immunoelectrophoresis of hemolymph samples from female neotenic (N; pair at left) and adult (A; pair at right) reproductives of *Z. angusticollis* on 0.8% agarose containing 3% antibody.

FIGURE 2. Schematic drawing of "fused rocket" immunoelectrophoresis of hemolymph antigens from female neotenic (N) and adult reproductives (A) and purified yolk protein from neotenic reproductives (Y) on 0.8% agarose containing 3% antibody.

Polyacrylamide gel electrophoresis

Partial characterization of TEAE-cellulose purified yolk was carried out by SDS-polyacrylamide gel electrophoresis following the methods of Weber, Pringle, and Osborn (1972). Phosphorylase a, pepsin, trypsin and lysosyme were used as molecular weight standards. Polyacrylamide gel electrophoresis was carried out in 3.5% gels with Tris-EDTA-Citrate buffer, pH 7.5. Gels were stained with Coomassie blue R250 and scanned using a Gilford 240 spectrophotometer.

Results

Identity of hemolymph and yolk antigens

Rocket immunoelectrophoresis of hemolymph samples collected from female neotenic and adult reproductives showed two precipitin peaks (Fig. 1). Hemolymph samples from female larvae and male larvae and from male neotenic and male adult reproductives showed no visible reaction with the antisera to crude yolk by this method. Fused rocket immunoelectrophoresis showed a reaction of identity of the neotenic hemolymph antigens with the TEAE-cellulose purified vitellins from neotenic reproductives and with hemolymph antigens from adult

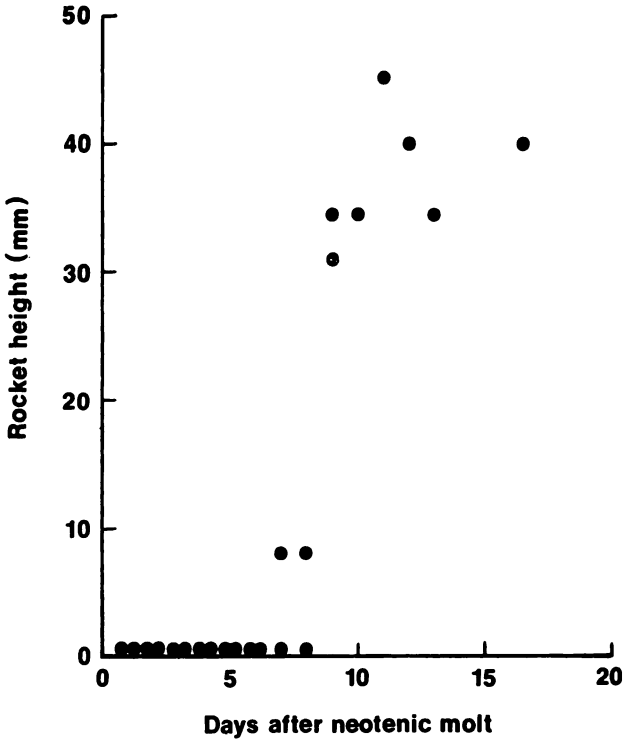


FIGURE 3. Relative concentration of vitellogenin in 1 ul hemolymph samples taken from female neotenic reproductives from day 1 to 18 days after the neotenic molt. Each point represents a sample taken from a different animal.

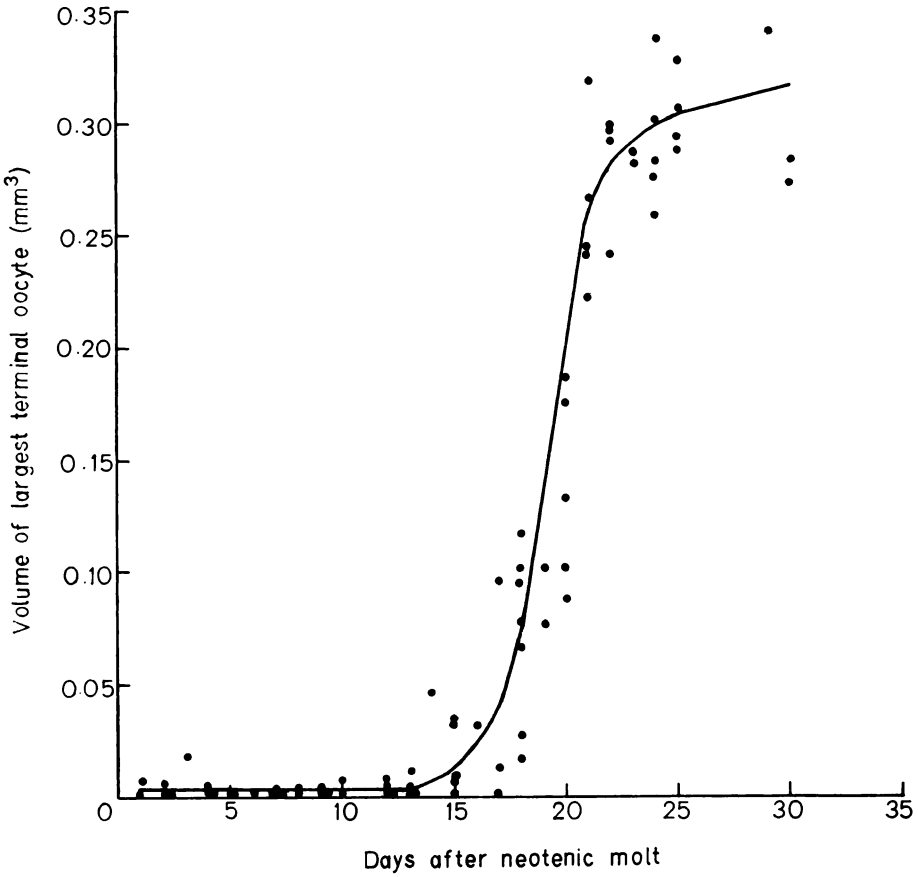


FIGURE 4. Volume (mm^3) of largest terminal oocyte from *Z. angusticollis* neotenic reproductives from day 1 to 30 days after the neotenic molt. Oviposition occurred between days 22 and 30 (mean day 25).

reproductives (Fig. 2), therefore the hemolymph derived antigens are immunologically identical with the vitellins and are aptly called vitellogenins.

Timing of hemolymph vitellogenin titre and oocyte growth

Hemolymph from female neotenic reproductives was not found to contain the vitellogenin until seven to nine days after the neotenic molt (Fig. 3). Ovaries from neotenic reproductives subsequently showed rapid growth between days 15 and 20, resulting in the oviposition of mature eggs between 22 and 30 days (mean 25 days) after the neotenic molt (Fig. 4). Over a period of three weeks, between 55 to 130 eggs were laid, or an average of 2.6 to 6.1 eggs per day. The accumulation of vitellogenin in the hemolymph is probably not derived from atretic follicles, since the vitellogenin appears in the hemolymph prior to any visible yolk deposition.

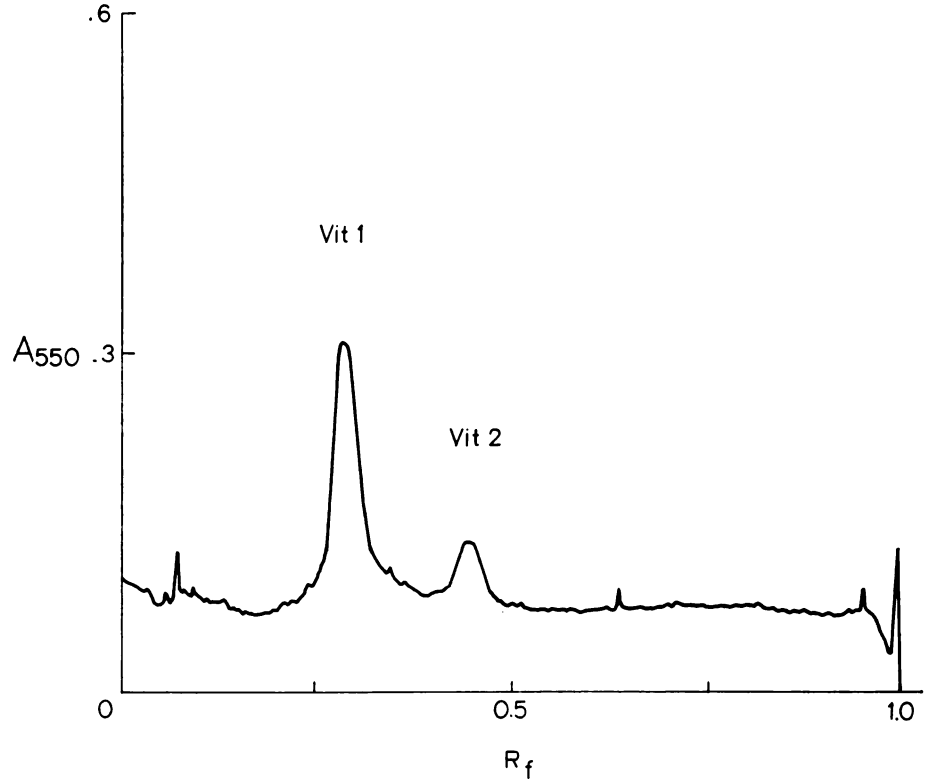


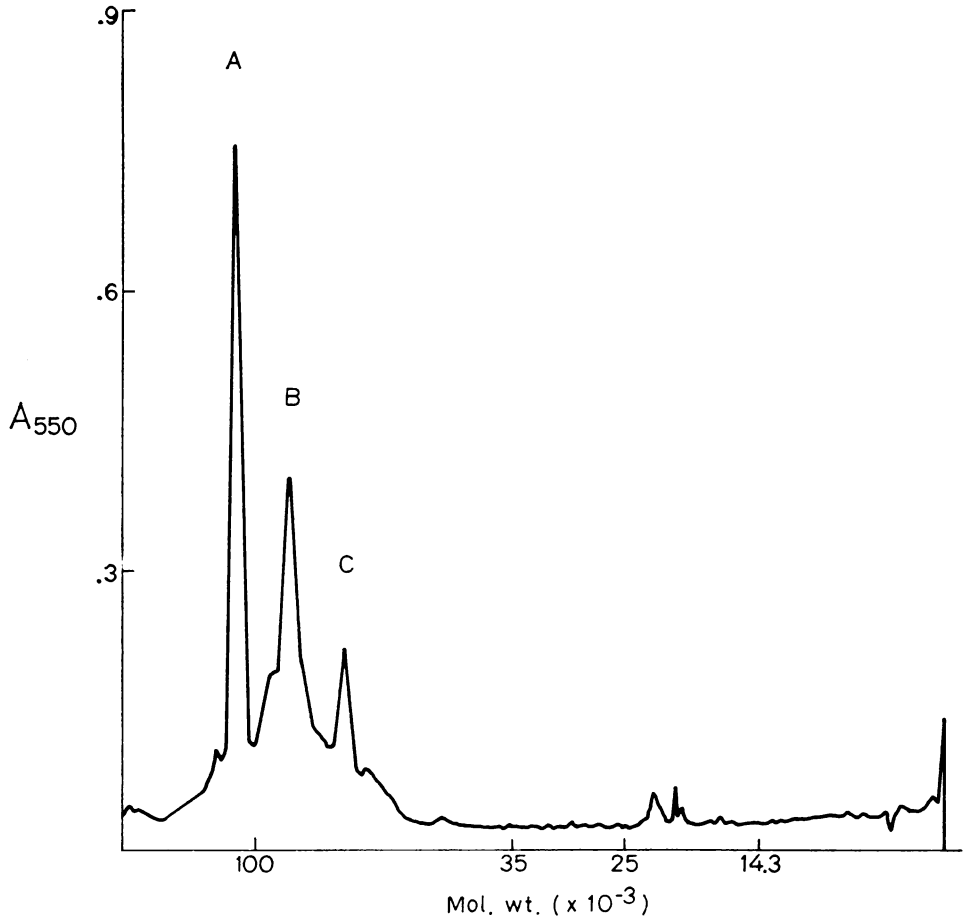
FIGURE 5. Gel profile of purified yolk protein from female neotenic reproductives separated by acrylamide gel electrophoresis (pH 7.5, 3.5% gel, Tris-EDTA-Citrate buffer), and showing the two vitellins (Vit. 1 and Vit. 2), using a Gilford Spectrophotometer (A_{550}). Proteins were stained with Coomassie blue R250.

Properties of termite vitellin

Gel electrophoresis of TEAE-cellulose purified vitellin from day-old neotenic reproductive eggs in nondenaturing conditions gave two distinct bands (Fig. 5), showing the existence of two distinct vitellins. Preliminary characterization of this same sample on SDS-gel electrophoresis gave three major bands (Fig. 6). The molecular weights of the two fastest traveling components were estimated to be 89,000 and 71,000 Daltons, respectively. The slowest traveling component did not travel as fast as phosphorylase a and was estimated by extrapolation at 112,000 Daltons.

DISCUSSION

The major yolk proteins, vitellins, of insects are normally found only in female reproductives and are transported to the ovaries via the hemolymph as vitellogenins. This report represents the first description of the female specific hemolymph proteins of a primitive termite, *Z. angusticollis,* and, indeed, of any of the Isoptera. The hemolymph proteins described in this report are undoubtedly vitellogenins



FIGURES 6. Purified yolk protein from female neotenic reproductives, separated by SDS-polyacrylamide gel electrophoresis (7.5% acrylamide). Molecular weights represent those of the standards phosphorylase a, 100,000; pepsin, 35,000; trypsin, 25,000; and lysosome, 14,300 Daltons. The molecular weights of the three major subunits were estimated to be A, 112,000; B, 89,000 and C, 71,000 Daltons.

since, as in other insects, they were found only in female reproductives, appeared in the hemolymph prior to oocyte growth, and became the major proteins in the mature oocytes. The hemolymph vitellogenins are, furthermore, immunologically identical to TEAE-cellulose purified vitellin obtained from eggs. The hemolymph vitellogenins are also immunologically identical in both the neotenic and adult reproductives of *Z. angusticollis*. Thus, there do not appear to be any specific differences in the vitellogenins produced by the two reproductive sub-castes of this social insect species. This is similar to what has been found for the honey bee *Apis mellifera* where the female specific protein of workers and queens is immunologically identical (Rutz and Lüscher, 1974).

The data demonstrate that in neotenic reproductives of *Z. angusticollis*, vitellogenin is first produced, or at least released into the hemolymph, seven to nine days

after the neotenic molt. It is not known what regulates vitellogenesis in this species, but nutrition or other colony interactions may play an indirect role; neotenic reproductives isolated soon after the molt from the parent colony and kept as heterosexual pairs had levels of hemolymph vitellogenin close to zero and even after 60 days had not laid eggs (Greenberg, 1978). These isolated neotenics do not receive the benefits of communal relations with other colony members, such as grooming and food exchange, which may be important for normal reproductive development. The control of ovarian development in neotenic and adult reproductives may not be entirely similar, however, in that, in contrast to neotenics, adult reproductives must leave the colony and become established as isolated pairs for oogenesis to occur normally (Hewitt and Nel, 1969). Ovary development in the adult reproductive appears to be inhibited by tactile stimuli received from other termites in the colony (Hewitt, Watson, Nel and Shoeman, 1972). There is evidence suggesting that ecdysone is ultimately necessary to stimulate oocyte maturation and protein synthesis in the ovary of the termite queen (Lüscher, 1976). Juvenile hormone, which plays a key role in cockroach vitellogenesis (Kunkel, 1973; Engelmann and Friedel, 1974), may not be necessary or may even have an inhibiting effect in termites (Lüscher, 1976). Indeed, preliminary attempts to induce vitellogenesis in *Z. angusticollis* neotenics by injection of juvenile hormone have not been successful (Greenberg, unpublished data). This lack of a role of JH would be surprising, however, considering the close relationship of cockroaches and termites (McKittrick, 1965; Kunkel and Lawler, 1974).

Preliminary characterization of the TEAE-cellulose purified yolk proteins of *Z. angusticollis* suggests that they are similar in solubility properties (being insoluble in low ionic strength media) to vitellin from other insects (Kunkel and Pan, 1976; Gellissen, Wajc, Cohen, Emmerich, Applebaum and Flossdorf, 1976; Engelmann, 1978) and are composed of several subunits. The assignment of the 3 major subunits to the two native vitellins is not yet clear, nor is the subunit composition known for the hemolymph vitellogenin. On the basis of immunological identity, however, it appears that the hemolymph vitellogenins and the egg yolk vitellins are indistinguishable. The presence of two hemolymph vitellogenins in this termite species is not surprising in view of the fact that multiple vitellogenins have been found in the cockroaches *Periplaneta americana* by Bell (1970) and *Brysotria fumigata* by Barth and Bell (1970). No other specific data of this nature is available for comparison, however, for any other of the Isoptera.

The site of synthesis of the termite vitellogenins described in this report is not known, though there is considerable evidence suggesting that in other insects the fat body is the site of synthesis of these proteins (Pan, Bell and Telfer, 1969; Hagedorn and Judson, 1972; Chen, Couble, Delucca, and Wyatt, 1976). Since the major egg yolk proteins described in this study are antigenically identical to proteins found in the hemolymph prior to oocyte growth, we are inclined to believe that vitellogenin production in this primitive termite, *Z. angusticollis,* takes place in the fat body, as in the majority of other insects studied. The data cannot, however, exclude the possibility that part or parts of the vitellin may be synthesized in the follicle cells. This statement is made in view of the fact that Wyss-Huber and Lüscher (1975) have presented evidence which they interpret as suggesting that the ovarian follicle cells are the main site of vitellogenin synthesis in queens of the higher termite *M. subhyalinus.* The queens of the large societies of the higher

termites are uniquely physogastric and are unsurpassed among the Insecta in the enormous quantities of eggs they are capable of producing. As a consequence of this, the entire structure of the abdomen and reproductive system of these queens may certainly have been modified in many as yet unknown ways. For instance, the enlarged ovaries of the higher termite queens occupy the major part of the abdomen and greatly distend it. The sternites and tergites become widely separated by the intersegmental membrane, whose tremendous increase in surface area is not yet understood (Weesner, 1969). Queens of the lower termites, however, do not generally become physogastric to the same extent as do those of the higher termites. Furthermore, the rate of egg laying is much lower. Neotenic queens in this study laid an average of between 2.6 and 6.1 eggs per day. Primary queens of the harvester termite *Hodotermes mossambicus* (Hagen) (Hodotermitidae) were reported to oviposit an average of between 1.3 and 7.5 eggs per day (Watson, 1972). It may very well be that in the higher termite queen the extremely rapid rate of egg production which results in the daily oviposition of tens of thousands of eggs is substantially enhanced by a follicle cell product. It should be pointed out, however, and taken note of that the methods of Wyss-Huber and Lüscher were not specific for termite vitellogenin, but simply measured relative rates of synthesis of unknown proteins by fat body and ovary. Their interpretations are therefore, at best, merely suggestive.

Among other eusocial insects of the Hymenoptera, most of the information available on vitellogenesis concerns species of the genus *Apis* (Reviewed by Engels, 1974). Queen honey bees can produce equal to their own weight in eggs daily and thus require correspondingly large amounts of vitellogenic proteins. It appears, however, that in *Apis mellifera* the yolk proteins are of extraovarian origin and that the oocyte itself, or the follicular epithelium, do not synthesize vitellogenins (Engels, 1974). Although the queen fat body is able to synthesize vitellogenin at a rate sufficient to explain its corresponding incorporation into eggs (Engels, 1972; cited in Rutz and Lüscher, 1974), some of the yolk precursor materials may be obtained from the workers (Engels, 1971, 1974; Rutz and Lüscher, 1974). The transfer of yolk precursor materials from worker bees to the queen could provide for the enhanced vitellogenesis needed for the high rate of egg laying which occurs during the active summer period. It is not known, however, if this type of supplementation can take place in termite colonies. The workers of higher termites are not true adult insects, as are those of the Hymenoptera, but are a terminal larval caste. Nevertheless, they do feed the physogastric queen, who is generally unable to feed herself. It is not known however, if workers of the higher termites produce or transfer vitellogenin precursor proteins to the queen.

In view of the potential importance of the results of Wyss-Huber and Lüscher, however, and the fact that in other insects vitellogenins have been found to be primarily of extraovarian origin, more specific studies are certainly needed to determine the site of synthesis of the termite vitellogenins.

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Summary

1. Vitellogenesis was studied in the primitive termite *Zootermopsis angusticollis*. Quantitative (rocket) immunoelectrophoresis was used to demonstrate two immunologically distinct sex-specific hemolymph proteins, vitellogenins, in female reproductives which were immunologically identical in neotenic and adult reproductive serum and in egg yolk from neotenic reproductives.

2. The vitellogenins are not detectable in hemolymph of neotenic reproductives by our methods until seven to nine days after the neotenic molt. A marked increase in oocyte volume was observed beginning six to eight days later and oviposition occurred approximately 25 days (range 22 to 30 days) after the neotenic molt. Presumably, vitellogenin production in this primitive termite occurs outside the ovary, as in the majority of other insects studied. However, the exact site of synthesis of this protein has not yet been demonstrated in the Isoptera.

3. Acrylamide gel electrophoresis at pH 7.5 of TEAE-cellulose purified egg yolk protein also demonstrated the existence of two distinct vitellins. SDS-gel electrophoresis of purified egg yolk protein showed that the two vitellins are composed of at least three subunits whose molecular weights were estimated to be 112,000, 89,000 and 71,000 Daltons.

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