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YOLK PROTEIN SYNTHESIS IN THE OVARY OF *OCTOPUS VULGARIS* AND ITS CONTROL BY THE OPTIC GLAND GONADOTROPIN

BY R. K. O'DOR* AND M. J. WELLS
Department of Zoology, University of Cambridge

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The synthesis of yolk proteins can conveniently be studied in *Octopus* where removal of the subpedunculate lobe from the brain causes secretion of a gonadotropin by the optic glands and results in precocious sexual maturation (Wells & Wells, 1959). Maturing animals can thus be prepared as needed. The normal reproductive pattern of the female is a further advantage. All the ova from any single animal are deposited during a short period (Mangold-Wirz, 1963) and therefore tend to develop synchronously (Brock, 1879). This results in a high overall rate of yolk synthesis and provides relatively uniform samples of vitellogenic ova without special selection.

Early studies of developing ova in cephalopods described the formation of a layer of follicle cells surrounding the oocyte and suggested that these cells were a site of synthesis of yolk material during secondary vitellogenesis (Lankester, 1875; Loyez, 1906; Yung, 1930). Konopacki (1933), however, reported the presence of lipoproteins in the blood sinuses outside the follicular epithelium. This evidence has been used in the recent literature (Arnold, 1971) to support analogies of cephalopods with species which produce yolk precursors in distant organs and transport them to the ova via the circulation. Among vertebrates and insects the liver (Wallace & Jared, 1969) and fatbody (Brookes, 1969), respectively, synthesize lipoprotein precursors which are incorporated into the developing ova with only minor modifications. In the experiments presented here the appearance of injected [^{14}C]leucine in protein in the liver, blood and ovaries of female *Octopus vulgaris* was followed to test this analogy. The results support the earlier view that in cephalopods the ovary itself is the major site of yolk synthesis.

The same series of experiments was used to examine the role of the optic gland gonadotropin in the control of vitellogenesis. The initiation of sexual maturation by the optic gland hormone is well established (Wells & Wells, 1959), and a continued requirement for it for maintenance of spermatogenesis has been shown both *in vivo* in *Octopus* (Wells & Wells, 1972) and in organ culture in *Sepia* (Richard, 1970). Using the same *in vitro* system Durchon & Richard (1967) found that oogonial multiplication, primary vitellogenesis and the proliferation of the follicle cells require the hormone; but they were unable to study ova advanced to the stage of secondary vitellogenesis. In our experiments removal of the optic glands prior to injection of [^{14}C]leucine prevented the incorporation of label into ovarian protein, suggesting that this later stage of vitellogenesis can also be added to the list of maturational processes controlled by the product of the single type of secretory cell (Wells & Wells, 1959; Nishioka, Bern & Golding, 1970) present in these glands.

* Present address: Department of Biology, Dalhousie University, Halifax, Canada.

The present study also describes a synthetic medium which will support a high rate of protein synthesis by isolated ovarian follicles. This synthesis confirms that the ovary is a major site of yolk production, and should provide a biological assay useful in further studies on the nature and mode of action of the optic gland hormone and perhaps other molluscan gonadotropins.

MATERIAL AND METHODS

Experimental animals and surgical procedures

Precocious maturation was induced in female *Octopus vulgaris* weighing 200–400 g by surgical removal of a region of the brain including the subpedunculate lobe. This procedure and the characteristic enlargement of the optic glands which follows has been described elsewhere (Wells & Wells, 1959). At the same time the muscle and connective tissue linking the floor of the mantle to the midline of the animal was cut. This operation does not significantly interfere with respiration and permits one to fold the mantle inside-out when the animal is relaxed under anaesthesia. This exposes most of the viscera and allows visual inspection of the ovaries, injections, removal of blood samples and surgical procedures to be carried out with minimal trauma after only brief exposure to a 3% urethane in seawater anaesthetic.

The animals were kept in the Zoological Station at Naples at 24 ± 2 °C for 17 days on a standard diet of about 10 g of crab or fish per day. At this time examination of the ovaries indicated that all had entered the stage of secondary vitellogenesis. Animals designated OG+ continued normal development for 3 days and were then starved for 2 days prior to injection of label. Animals designated OG− had the optic glands removed at 17 days, but were otherwise treated in the same manner as the OG+ group. Control animals were either unoperated or sham-operated (brain exposed, but nothing removed), and were kept under the same conditions as the experimentals.

Injection, sampling and radiometric techniques

Uniformly labelled [^{14}C]leucine with a specific activity of 342 mCi/mmol was supplied by The Radiochemical Centre, Amersham as a 50 μCi/ml aqueous solution. A 1:3 dilution of this material in sea water was injected into anaesthetized animals via the left branchial heart (see Fig. 3) in quantities as indicated in the results, and they were revived by running sea water over the gills. At the appropriate time(s) they were re-anaesthetized and blood samples were collected from the right branchial heart for counting. After obtaining blood samples, animals to be sacrificed for tissue samples were bled for about 10 min through a canula placed in the dorsal aorta to reduce the quantity of blood present in the tissues. The ovary, liver and other tissue samples required were then removed, kept separately and frozen.

Duplicate 0·10 ml blood samples were dried on 2·1 cm discs of Whatman GF/A glass fibre paper and left overnight in 1·0 ml of 0·1 M-L-leucine in a 10% (w/v) solution of trichloroacetic acid (TCA) at 4 °C. 0·50 ml aliquots of the TCA solution were diluted to 1·0 ml with distilled water and counted in 10·0 ml of either Triton X-100, toluene (1:2), 0·8% (w/v) 2(4′-*t*-butylphenyl)-5-)4″-biphenylyl)-1,3,4-oxadiozole scintillation fluid or Instagel (Packard Instrument Co.). Counts per minute were determined in a Packard Tri-Carb scintillation spectrophotometer and suitable

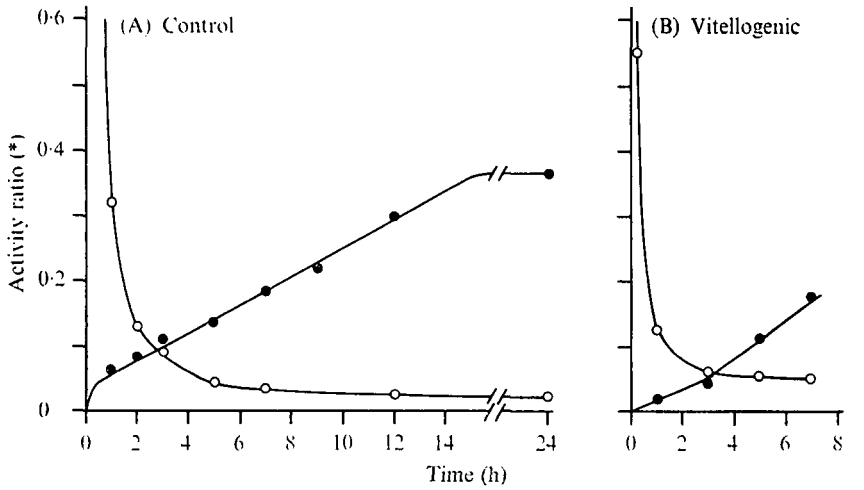

Fig. 1. The levels of radioactivity in the TCA-soluble and protein fractions of sequential blood samples from normal (A) and precociously mature (B) female octopuses following injection of [^{14}C]leucine. ○, TCA-soluble fraction, ●, protein fraction.

* The activity ratio is the DPM per ml of blood divided by the DPM injected per g body weight (see text).

corrections for quenching were made to convert to disintegrations per minute (DPM). These TCA-soluble counts were used as an index of non-protein label remaining in the blood. The glass discs were then processed to eliminate non-protein material as described by Mans & Novelli (1961) and counted in 5·0 ml of 0·3 % (w/v) 2,5-diphenyl-oxazole in xylene. Corrections to DPM were made by comparison to standards of [^{14}C]leucine added to 0·10 ml of unlabelled blood dried on discs. The average values from duplicate samples were used to calculate levels of labelled protein in the blood.

Duplicate samples of known weights between 0·1 and 0·2 g from frozen tissues were crushed in 1·0 ml of 0·1 M-L-leucine in 10% (w/v) TCA and left overnight at 4 °C. 0·50 ml aliquots of supernatant were counted as above to determine soluble counts. After processing as described by Greengard *et al.* (1964), the remaining protein fraction was hydrolysed for 4 h at 100 °C in 1·0 ml of 2 M-NaOH and acidified with 1·5 ml of 2 M-HCl. 1·0 ml aliquots were counted without dilution in Triton-toluene or Instagel scintillation fluid as described above and corrected for quenching. The averages of duplicate samples were used to calculate levels of labelled tissue protein.

Where different animals are compared a quantity called the 'activity ratio' is used. This is the ratio of the DPM per gram of tissue or per millilitre of blood to the total DPM injected per gram body weight and allows corrections for variation in animal weight and for [^{14}C]leucine losses which occasionally occurred during injection. These losses were monitored by counting aliquots of the sea water in the container used to revive the animals.

RESULTS

Uptake of labelled leucine and its incorporation into protein

In preliminary experiments a 750 g control and a 380 g OG+ animal were injected with 15·0 and 5·0 μCi, respectively, of [^{14}C]leucine; and sequential blood samples were taken at intervals as indicated in Fig. 1. Assuming a blood volume of 5·8 ± 1·0% of the

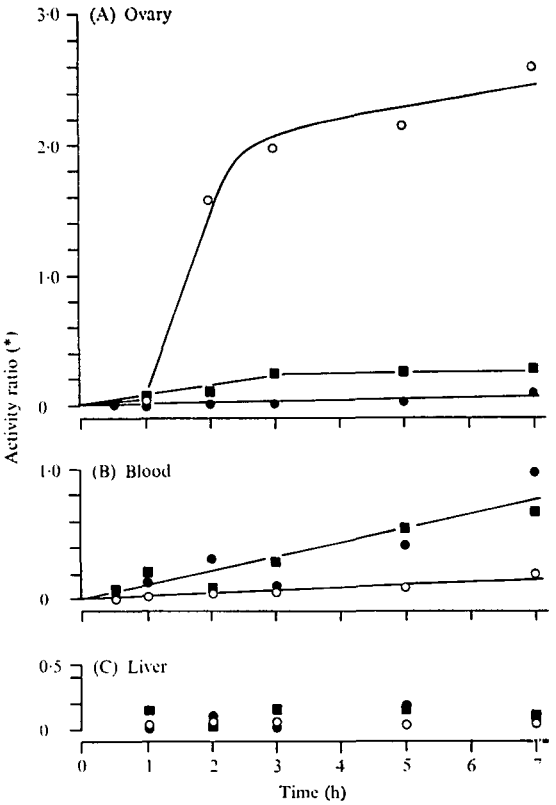

Fig. 2. The levels of radioactivity in the protein fractions of the ovaries (A), blood (B) and livers (C) of female octopuses in three different physiological states sacrificed at intervals following injection of [^{14}C]leucine. ■, normal, ○, precociously mature (OG+), ●, precociously mature, but with optic glands removed (OG−) 5 days prior to injection.
* The activity ratio is the DPM per ml of blood or per g of tissue divided by the DPM injected per g body weight (see text).

body weight as estimated for *Octopus dofleini* (Martin *et al.* 1958) the dose of [^{14}C]-leucine given to this control animal should give an initial activity in the blood of 765000 DPM/ml. The TCA-soluble DPM in the blood sample collected after 1 h (Fig. 1A) was 14300 DPM/ml or 1·9% of the expected initial activity. The comparable figure for the OG+ animal was 0·6% and a sample collected after 10 min showed only 3·2%. The disappearance of the label from the blood is thus quite rapid, and the injected dose should behave as a pulse since the labelled amino acid is only available from the blood for a short period.

If yolk precursors were synthesized at a high rate in the liver and released into the blood for uptake by the ova, one would expect to find an early rise in labelled protein, followed by a decline as the pulse of amino acid is used up. The sequential blood samples from the OG+ animals shown in Fig. 1B do not show this pattern although the animal was clearly in the stage of secondary vitellogenesis and nearly 15% of the total label injected was present as protein in the ovary at the end of the 7 h experiment.

Further information on the ovarian prtoein and its source was obtained by injecting a series of control, OG+ and OG− animals (6 of each type) with 3·3 μCi of [^{14}C]-

Table 1. *Details of ovary and body weights and of labelled protein distribution for animals sacrificed after 5 h*

Group	Animal wt (g)	Ovary wt (g)	Protein DPM/Total DPM injected × 100		
			Ovary	Blood*	Liver
Control	352	0·6	0·1	3·2	1·9
OG− †	296	18	0·2	2·5	0·6
OG+ †	254	45	38	0·5	0·1

* Assuming blood volume as reported by Martin *et al.* (1958). 5·8 ± 1·0 % of body weight.
† As defined in Fig. 2 and text.

leucine and sacrificing them after intervals of from one half to 7 h as indicated in Fig. 2. All three groups showed a progressive rise in labelled blood protein (Fig. 2B), but again there was no indication of an early rise and fall. The rates of accumulation were similar in the control and OG− groups, but considerably lower in the OG+. Ovarian protein, however, increased dramatically in the OG+ animals between 1 and 2 h after injection (Fig. 2A) in marked contrast to the OG− group which showed essentially no incorporation of label into protein in the ovaries. The smaller ovaries of the controls showed some incorporation, but this is exaggerated by the activity ratio used in Fig. 2. Table 1 better illustrates the differences in the quantities of label involved in the three groups in relation to the total [^{14}C]leucine injected. The levels of labelled protein in the livers of all three groups were low and relatively stable (Fig. 2C).

It is clear from these results that large quantities of protein are rapidly accumulated by the maturing ovaries of the OG+ animals and that removal of the optic glands stops this process. The low levels of labelled protein in the liver of all groups and the absence of fluctuations in these levels seem to rule out a role for this organ in the synthesis of the ovarian protein. The low levels of labelled blood protein in the OG+ animals might appear to suggest that protein was being removed from the blood by the ovary, but in fact the quantity of labelled protein in the blood could only account for a small fraction of that in the ovary. The protein fraction of the ovary of the 2 h OG+ animal contained 833000 DPM while the average concentration of labelled protein in the blood of the 1 h animals was only 570 DPM/ml. The ovary would thus have had to clear 1450 ml of blood between the first and second hours to accumulate the observed quantity of label. We have not measured blood flow through the ovary, but Johansen (1965) reported the resting cardiac output of *Octopus dofleini* to be 10 ml/kg/min when the oxygen consumption was about 20 ml/kg/hr. Since the blood of both *O. dofleini* (Martin *et al.* 1958) and *O. vulgaris* (Craifalaenau, 1919) contain about the same concentration of the oxygen-carrying protein, haemocyanin, (~ 10 g/100 ml) it is likely that the cardiac output will be proportional to the oxygen consumption, which Montouri (1913) reported as 60–90 ml/kg/hr in *O. vulgaris*. Values of about 100 ml/kg/h were reported more recently for *O. cyanea*, a species similar in size and habit to *O. vulgaris* (Maginniss & Wells, 1969). Using this highest figure the calculated cardiac output is 50 ml/kg/min, or for the 200 g animals in this experiment a total of 600 ml in 1 h. Thus even if the entire cardiac output passed through the ovary and

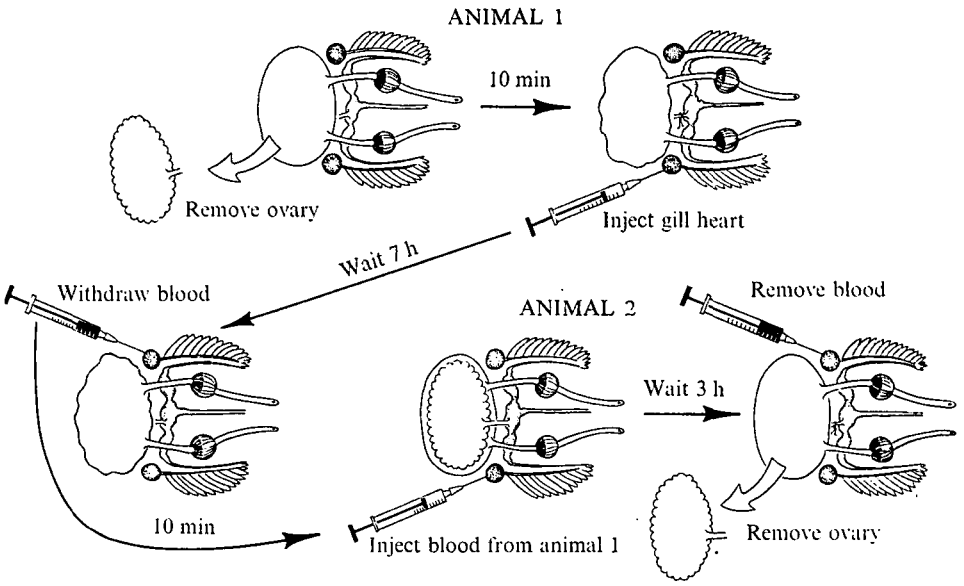

Fig. 3. Pattern of transfusion experiments to determine whether blood proteins are incorporated into the ovary. Labelled blood proteins were produced by ovariectomized donors and transfused to precociously mature recipients.

100% of the labelled protein were removed as it passed, it would still account for less than half of the labelled ovarian protein.

Transfusion from ovariectomized animals

In the experiments above it is conceivable that a large peak of labelled protein passed through the blood between samples or that a fraction of the ovarian protein was being taken up from the blood. Either of these phenomena should result in a marked accumulation of labelled blood protein in ripening animals ovariectomized immediately prior to injection of [^{14}C]leucine. Two OG+ animals were ovariectomized by making a small incision through the wall of the gonadial coelom, and squeezing out the egg mass. A ligature was tied around the genital aorta and vein. The empty ova sac was left in place and animals were injected with [^{14}C]leucine. After 7 h a 1–2 ml blood sample was taken from each animal and 0·10 ml aliquots used for counting as before. These samples had activity ratios of 0·40 and 0·45, higher than the OG+ animals in Fig. 2B but lower than either the OG− or control animals. The soluble DPM in each sample was less than 5% of the protein DPM and did not contribute significantly to later results.

Despite the low levels of labelled protein present the remainder of each sample was injected into an OG+ recipient with maturing ova, as shown in Fig. 3. If the blood protein were yolk material *en route* to the ovary one would expect it to accumulate there within 2–3 h of injection (see Fig. 2A) but again the results failed to show a relationship between the blood protein and protein in the ovary. Three hours after injection of the labelled blood the level of protein DPM indicated that 82 and 110% of the injected protein was still present within the blood of the two recipients. The total quantity of label in the blood was calculated assuming a blood volume of 5·8%

of body weight, and the deviation from 100% is within the variation of blood volume (±1·0%) reported by Martin *et al.* (1958). The level of labelled protein in the ovaries was no more than that to be expected from their blood content alone.

It should be noted that the recovery following ovariectomy was excellent and that the animals resumed feeding the following day. There was no evidence of ill effects from the operation during the 10 days that the animals were kept.

In vitro *synthesis by ovarian follicles*

As final confirmation that the follicles themselves are a major site of protein synthesis, ovarian follicles still attached to their stalks were collected from OG+ animals, blotted dry on filter paper and weighed. Approximately 0·1 g of tissue was added to 1·0 ml of medium and incubated on a shaker for 6 h at 25 °C. The medium used was similar to that of Necco & Martin (1963) and was prepared by addition of 9·2 ml of sterile 290 mg/ml NaCl and 2·0 ml of 5000 unit/ml penicillin–streptomycin to 100 ml of medium 199 with Hank's BSS and Hepe's buffer. Medium 199 and antibiotics were obtained from Bio-cult Laboratory, Glasgow. [^{14}C]leucine was added to a concentration of 0·125 μCi/ml—0·16% of the total leucine concentration (230 μM) in the medium. 10 ovary samples from two OG+ animals were used and the average DPM (± standard error) present in the protein fractions of these samples after 6 h was 301 ± 21 DPM/mg fresh tissue. The protein fraction of a 100 mg sample thus contained 11% of the total [^{14}C]leucine present in the medium despite the high levels of 'cold' leucine. Assuming that the yolk protein is 8% leucine this is a synthesis rate of 1·7 mg of protein/g tissue/day. If, as earlier workers suggested and our own cytological studies imply (Buckley, Wells & Wells, in preparation), this synthesis occurs in the follicle cells which make up about 5% of the mass of the ovary at this stage, these cells are producing protein at a rate of 30 mg/g cells/day. This is lower than the maximum *in vivo* synthesis rate of about 200 mg/g cells/day estimated from the increase in ovary weight assuming a protein concentration of 25% in yolk (Fugii, 1960), but confirms the *in situ* synthesis in the ovary. These are both high synthesis rates but not unreasonable when compared with the 250 mg casein/g tissue/day produced in rat mammary gland (Hanwell & Linzell, 1972).

DISCUSSION

The ova in *Octopus* are relatively large and rich in yolk. The evidence presented here indicates that when yolk deposition is occurring a large percentage of the circulating amino acids is destined for incorporation into protein in the ova. Protein synthesis does not occur at the required rate in the liver, and the protein which accumulates in the blood is not taken up by the ovary. It is therefore likely that in *Octopus* yolk proteins are produced in the ovary itself rather than as blood-borne precursors analogous to those seen in vertebrates and insects. The ability of isolated ovarian follicles to synthesize protein *in vitro* supports the view of earlier workers that the follicle cells are responsible for yolk synthesis during the period of secondary vitellogenesis. Cytological examination of the follicle cells of squid have shown that they are equipped for such synthesis during this period (Cowden, 1968), and our own

ultrastructural studies indicate that this is also the case in *Octopus* (Buckley, Wells & Wells, in preparation).

The blood protein seen in these experiments is probably haemocyanin. This hypothesis is supported by preliminary analyses of the control group's branchial glands, organs recently reported by Dilly & Messenger (1972) to be a source of haemocyanin on the basis of their ultrastructure. The protein activity ratio in this tissue rose to a maximum of 9·5 and fell to 3·5 7 h after injection of [^{14}C]leucine, suggesting a high rate of protein synthesis and release. The branchial glands weigh 0·5 to 1·0 g and at the observed activity ratios the quantities of labelled protein they contain would be adequate to supply the observed labelled blood protein.

An additional anomally relating to the blood protein is the depressed rate of accumulation seen in OG+ animals as compared with control and OG− animals in both the sequential (Fig. 1) and multiple animal (Fig. 2B) experiments. This could merely reflect the large amounts of label sequestered in the ovary, but as the ovariectomized OG+ animals also showed less accumulation in the blood than controls the effect may be more complex. The growth rate of *Octopus* during sexual maturation is lowered (Mangold-Wirz, 1963; Mangold & Boletzky, 1973) and may become negative (van Heukelem, 1973). Sakaguchi (1968) has reported a decline in digestive enzymes in the liver and salivary glands during this period and we have observed that the bases of the arms become unusually thin in maturing specimens. All of these facts suggest a decreased rate of protein synthesis outside the ovary in animals with active optic glands. The possibility that this is a direct effect of the optic gland hormone is being investigated.

The evidence presented clearly indicates a role for the optic gland hormone in the maintenance of yolk synthesis during secondary vitellogenesis, in addition to previously described effects on young oocytes and proliferating follicle cells. The follicle cells probably synthesize the yolk protein and are a likely target for the hormone. The ability of the ovarian follicles to carry out protein synthesis *in vitro* provides a convenient system for further studies on the mechanism of action of the *Octopus* gonadotropin and should provide a biological assay suitable for use in its isolation and chemical characterization.

SUMMARY

1. Over 98% of a dose of [^{14}C]leucine injected into the circulation of *Octopus vulgaris* is removed from the blood during the first hour.

2. There is a rapid accumulation of labelled protein in the ovaries of maturing animals within 2 h of injection. Within 5–7 h the ovaries contain nearly 40% of the injected label in protein form.

3. Removal of the optic glands prevents this accumulation of protein.

4. There is little labelled protein in the livers of either control or maturing animals at any time; but a slow, steady accumulation occurs in their blood.

5. The level of labelled protein appearing in the blood of acutely ovariectomized, maturing females is no higher than in controls; and when blood protein from ovariectomized animals is injected into normal maturing females it is not taken up by the ovaries.

6. The labelled protein which accumulates in the blood is probably haemocyanin. Preliminary experiments indicate that the branchial glands, which are already believed

to be a site of haemocyanin synthesis on morphological grounds, show a high rate of protein synthesis and release.

7. Isolated ovarian follicles in a liquid medium synthesize protein at a rate somewhat lower, but comparable with, the apparent *in vivo* rate.

8. The combined evidence from these experiments indicates that in *Octopus* yolk proteins are formed within the ovary – probably by the follicle cells – rather than being synthesized elsewhere and transported through the blood, as in arthropods and vertebrates.

9. The optic gland gonadotropin is essential for maintenance of protein synthesis during secondary vitellogenesis and the follicle cells are a likely site for its action during this stage of development.

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