Reprod. Nutr. Dévelop., 1983, **23** (1), 93-100.

Diurnal rhythms in the synthesis and release of haemolymph proteins in the crustacean Isopoda, *Porcellio dilatatus* (Brandt), with special reference to vitellogenin

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Summary. Synthesis and release of vitellogenin in the crustacean Isopoda, *Porcellio dilatatus,* were maximal during premolt. At that time, diurnal variations occurred in the haemolymph protein level and in the synthesis and release of vitellogenin and other proteins in the haemolymph. The pattern of variations was always identical and bimodal : the minima were at dusk and at dawn when the maxima occurred in the middle of the day and of the night. These variations were highly significant, and the diurnal means were significantly higher than the nocturnal means.

Introduction.

Oocyte yolk inclusions in insects are composed of an endogenous fraction and an exogenous fraction (see review by Engelmann, 1970). In several crustacean isopods, this inclusion takes place particularly in stage 2 of vitellogenesis ; in *Porcellio dilatatus* oocyte volume increases considerably (250 µm to 700 µm according to Besse, 1976) during this stage.

In *Idotea balthica* the incorporation processes, demonstrated at ultrastructural level by numerous images of pinocytosis in the oolemma of vitellogenic oocytes, are concomitant with enlargement of the spaces between follicular cells (Souty, 1980). This process permits the vitellogenin, formerly called « female-specific protein » in *P. dilatatus* and *Ligia oceanica* (Besse and Mocquard, 1968), to penetrate inside the oocytes. Vitellogenin, the precursor of intraoocyte yolk protein (Junéra and Meusy, 1982), is antigenically indistinguishable from it (Kerr, 1969 ; Croisille *et al.*, 1974 ; Picaud, 1978). It represents the major part of the exogenous yolk fraction and its incorporation is specific, at least in insects (Telfer, 1960 ; Kunkel and Pan, 1976 ; Ferenz, 1978 ; Ferenz *et al.*, 1981). The fat body constitutes the extraovarian source of vitellogenin in *P. dilatatus* (Picaud and Souty, 1980), *I. Balthica* (Souty and Picaud, 1981) and *Orchestia gammarellus* (Junéra and Croisille, 1980). According to Picaud and Souty (1981), the synthesis

and release of vitellogenin by the fat body of *P. dilatatus* undergoes changes during the molt cycle, being maximal in premolt, *i.e.* in the D_1', D_1'' and D_2a stages of Drach and Tchernigovtzeff (1967) adapted to *P. dilatatus* by Noulin (personal communication).

At these stages of the molt cycle we investigated putative diurnal changes in haemolymph protein levels and in synthesized and released vitellogenin and total protein over a given period.

Material and methods.

We used virgin females having the same origin and weighing between 90 and 130 mg ; they were the issue of a group reared under natural photoperiod at 20 °C. At the end of January the females were isolated and submitted to a photoperiod of 14L : 10D. After they had been with males for one week, 95 % of them began to reproduce. In our experimental conditions, they were able to carry out several successive vitellogeneses. According to Mocquard *et al.* (1976), these females were in molt stages D_1' to D_2a 9 to 13 days after pullus was released.

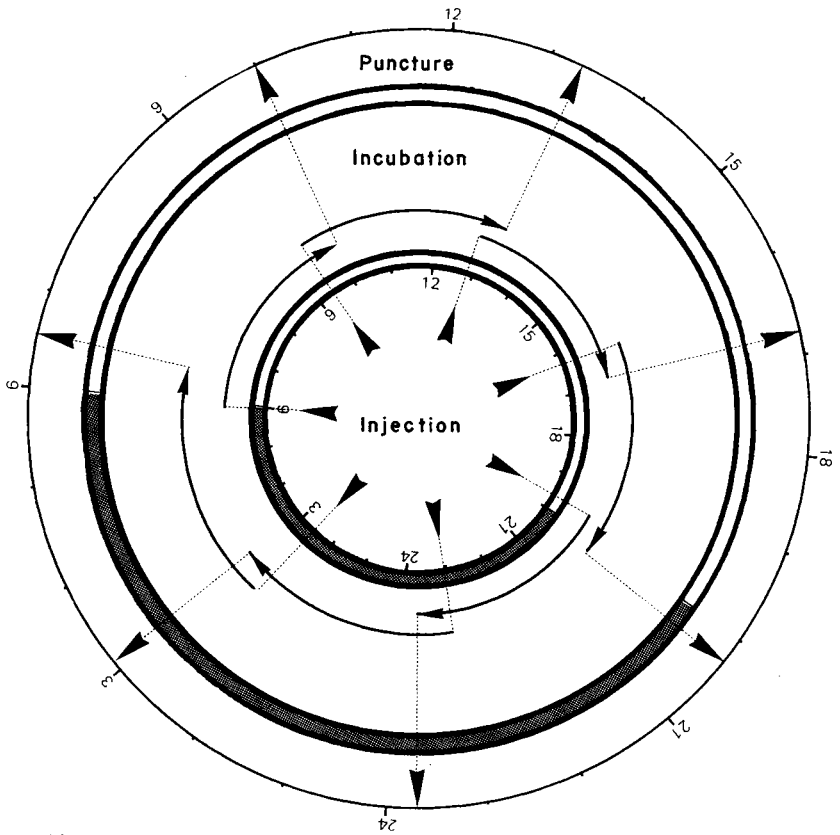

FIG. 1. — *Time schedule used in radioactive labelling of haemolymph protein.* Numbers : times of day. Black and white circles : photoperiod (14L : 10D).

At these presumed stages, several groups of females were tested at 7 points during the 24-hour period (fig. 1). To radioactively label the haemolymph proteins, each female was given an injection of 0.2 µCi of ^{14}C-leucine (Amersham France ; specific activity = 350 mCi/mmole) contained in a volume of 4 µl. Four hours after injection, 9 µl of haemolymph were withdrawn from each female by puncture (fig. 1) and diluted in 600 µl of physiological solution.

For treatment, we used the protocol described by Souty and Picaud (1981), Picaud and Souty (1981) and summarized on figure 2. The antivitellogenin antiserum was prepared in rabbit by injecting an extract of ovaries in early stage 2 of vitellogenesis in which the vitellogenin and the vitellin composed the major part of the ovarian proteins (Picaud and Besse, 1973). This extract was rendered monospecific against those proteins by absorption with male haemolymph.

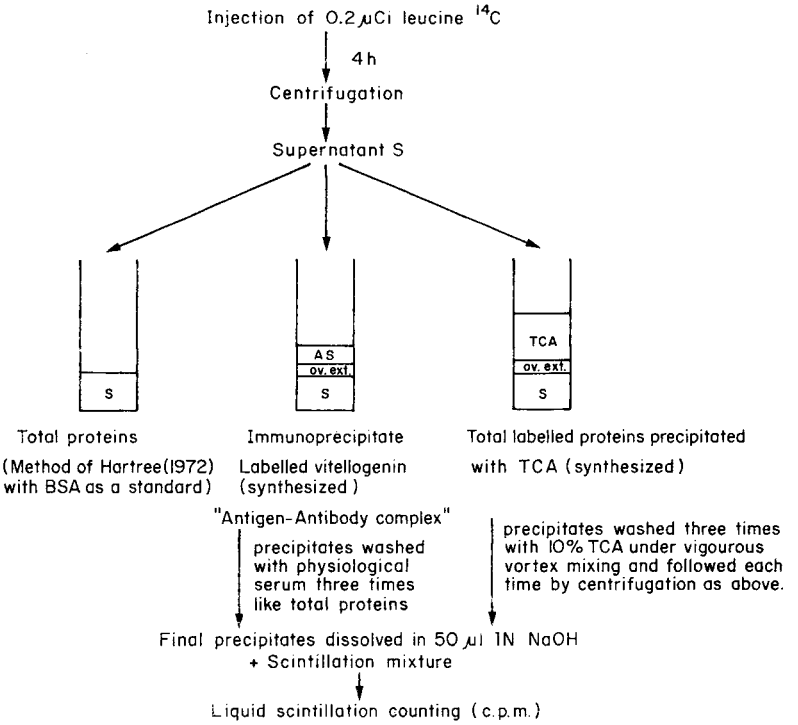

FIG. 2. — *Methods of protein analysis.*
S = three samples of 100 µl each ; AS = antiserum. The amount of AS was determined by titration curves and microtests. AS added, incubated for 1 h at 30 °C and then overnight at 4 °C. ov. ext. = ovarian extract ; TCA = trichloracetic acid.

Total unlabelled proteins were measured by the method of Hartree (1972) at the time of puncture and expressed in µg of equivalent albumin.

The labelled proteins and vitellogenin were expressed in CPM per µl of haemolymph, thus giving the quantities synthesized during the 4 hrs between injection and puncture.

At the end of each manipulation, 2 pereiopods were sampled, fixed, dehydrated and mounted to exactly determine molt cycle stage.

Results.

1. *Haemolymph protein level.* — Figure 3 shows data obtained on the levels of haemolymph protein. The lowest value occurred at the beginning of the dark phase ; the level then rapidly increased during the night and more slowly after daybreak, reaching a maximum at midday. This maximum was 75 % higher than the minimum observed 7 h later. Protein level heterogeneity was significant at the 5 $^{0}/_{00}$ level. The diurnal and nocturnal mean levels showed a significant difference at the 1 % level, the diurnal mean being 22 % higher than the nocturnal mean.

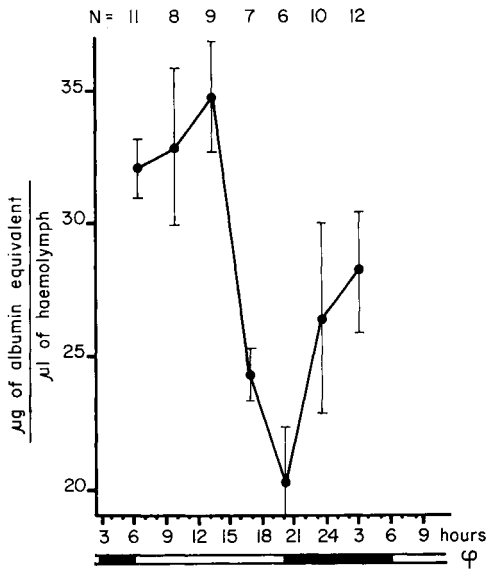

FIG. 3. — *Haemolymph protein level.*
N = number of animals tested ; φ = photoperiod (14L : 10D).

2. *Synthesis and release of total haemolymph protein.* — As the haemolymph protein level, that of the labelled protein of the haemolymph showed a minimal value at the beginning of the scotophase. Then, these synthesized and released proteins increased continuously during the night (fig. 4).

They were low in the early morning but maximal in the late morning. This maximum was 7 times higher than the minimum and diurnal fluctuation was significant at the 1 $^{0}/_{00}$ level. The diurnal mean was 70 % higher than the nocturnal mean, the difference being significant at the 1 $^{0}/_{00}$ level.

3. *Vitellogenin synthesis and release in the haemolymph.* — The fluctuation of labelled haemolymph vitellogenin (fig. 5) during the 24-hour cycle was the same as that of the labelled haemolymph protein. The late morning maximum represented 13 times the minimum situated in the early night. The fluctuation of synthesized and released vitellogenin, significant at the 1 $^{0}/_{00}$ level, was thus considerable. Compared to the diurnal and nocturnal means, the difference was significant at the 5 $^{0}/_{00}$ level with a diurnal mean 33 % higher than the nocturnal mean.

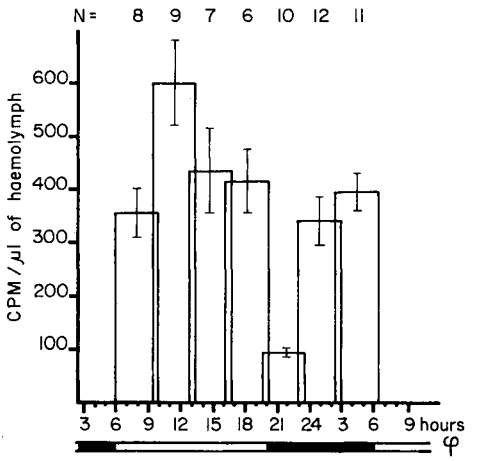

FIG. 4.

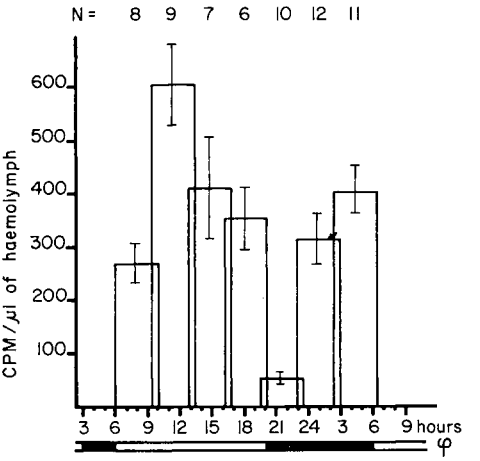

FIG. 5.

FIG. 4. — *Synthesis and release of total protein in the haemolymph.* Same symbols and conventions as figure 3.

FIG. 5. — *Vitellogenin synthesis and release in haemolymph.* Same symbols and conventions as figure 3.

Labelled vitellogenin, expressed in percentage of labelled total protein, also showed bimodal fluctuation during the day (significant at the 1 $^{0}/_{00}$ level) with a minimum of 57 % situated in the early night. Its highest value (99 %) occurred in the late night and the day-night difference was not significant at the 5 % level.

Discussion.

The data presented here demonstrate that the release of vitellogenin in *P. dilatatus* follows a diurnal rhythm in late vitellogenic females and in premolt. This large-amplitude rhythm, which must be considered in all studies on vitellogenin metabolism, nevertheless does not disagree with the results of Picaud and Souty (1981) cited previously since the studies were always carried out at the same hour.

During the 24-hour cycle the haemolymph protein level changed in the same way as did the synthesis and release of vitellogenin. However, according to electropherograms of vitellogenic females of *P. dilatatus* (Picaud and Besse, 1973), vitellogenin may represent 5 to 20 % of the total protein. The proportion of vitellogenin to total haemolymph protein in insects is situated within these limits ; 5 % in *Diploptera punctata* (Mundall, Tobe and Stay, 1981) and 25 % in *Locusta migratoria* (Ferenz, 1978).

Consequently, the amount of vitellogenin does not appear to be large enough to explain the fluctuations of total protein by vitellogenin release according to diurnal rhythm. However synthesized and released vitellogenin represents at least 57 % and, on an average, 80 % of synthesized protein released in the haemolymph between injection and puncture. Thus, it is probable that a large part of the total proteins released at the rhythm we found, is not labelled, *i.e.* they were synthesized more than 4 h before they were released.

Wieser, Schweizer and Hartenstein (1979) studied protein catabolism in two crustacean isopods, *Porcellio scaber* and *Oniscus asellus.* According to these authors who used the males of these species, ammoniac excretion, reflecting protein turnover, follows a diurnal rhythm homologous to the one we observed. This would confirm the hypothesis of a protein-releasing rhythm in *P. dilatatus,* as described previously.

On the other hand, diurnal changes in the enzymatic activities of *Penaeus kerathurus* (Van Wormhoudt, Ceccaldi and Le Gal, 1972) are bimodal and approximately the reverse of the rhythms we found. The same phenomenon occurs for some enzymes of the abdominal muscle of *Palaemon squilla* (Trellu and Ceccaldi, 1977).

Finally, Chentoufi (1982) recently studying *Armadillidium vulgare* (Crustacea, Isopoda) showed a circadian rhythm for breathing that was similarly bimodal and in reverse phase with the rhythms of protein release in *P. dilatatus.*

Conclusion.

Most of the physiological rhythms observed in this study present either maxima or minima at the borderline between day and night and inversely, indicating dependence on photoperiodic rhythms.

On the other hand, as the photoperiod is the principal external factor controlling reproduction in isopods (*P. dilatatus* : Mocquard *et al.,* 1978 ; *Armadillidium vulgare* : Mocquard *et al.,* 1980), it is possible that diurnal rhythms have direct control over reproduction.

The existence of such a rhythm offers a new field of investigation concerning vitellogenin. Moreover, vitellogenin, which is the object of much study, seems to be suitable for investigating diurnal rhythm control.

Reçu en juillet 1982.
Accepté en août 1982.

Acknowledgements. — We wish to thank L. Nasreddine for typing the paper.

Résumé. *Rythme nycthéméral de synthèse et de libération de la vitellogénine et des protéines hémolymphatiques chez le crustacé Isopode terrestre,* Porcellio dilatatus *(Brandt).*

Dans l'hémolymphe de l'isopode terrestre *Porcellio dilatatus,* le niveau maximum de synthèse et de libération de vitellogénine est atteint en prémue. A cette période du cycle de mue, on observe des variations nycthémérales du taux des protéines hémolymphatiques, de la synthèse et de la libération de vitellogénine et des autres protéines dans l'hémolymphe. Dans tous les cas, le schéma de variation est identique et bimodal : au crépuscule et à l'aube se situent les minimums, au milieu du jour et de la nuit les maximums. Les variations observées sont hautement significatives et les moyennes diurnes sont significativement supérieures aux moyennes nocturnes.

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