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Original article

In vitro effect of the gonad of *Helix aspersa* (Mollusca) on galactogen synthesis in the albumen gland of either mated or virgin snails

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Summary — The gonad of *Helix aspersa* contains a factor which can stimulate in a dose-dependent manner galactogen synthesis in albumen gland explants cultured *in vitro*. The stimulatory activity appears to be greater when the gonad is predominantly male than when it is predominantly female. The albumen gland of virgin snails does not respond *in vitro* to the gonadal influence. The receptivity of the albumen gland to the galactogen synthesis stimulating effect of the gonad is increased after the first and second mating. It decreases at the third mating in correlation with the increase of the albumen gland maturation index.

***Helix aspersa* / albumen gland / galactogen / gonad / mating**

Résumé — Effet *in vitro* de la gonade de *Helix aspersa* sur la synthèse de galactogène de la glande à albumen d'escargots vierges ou accouplés. *La gonade de* Helix aspersa *contient un facteur capable de stimuler, d'une façon dépendante de la dose, la synthèse de galactogène dans des explants de glande à albumen cultivés* in vitro. *L'activité stimulante apparaît plus importante dans la gonade en phase mâle dominante que dans la gonade en phase femelle dominante. La glande à albumen d'escargots vierges ne répond pas à l'influence gonadique* in vitro. *La réceptivité de la glande à albumen à l'effet stimulant de la gonade sur la synthèse de galactogène est augmentée après le premier et le deuxième accouplement. Elle diminue au troisième accouplement corrélativement à l'augmentation de l'indice de maturation glandulaire.*

Helix aspersa / *glande à albumen / galactogène / gonade / accouplement*

INTRODUCTION

In pulmonate molluscs, the albumen gland, one of the female accessory sex organs, secretes perivitelline fluid around the fertilized eggs (May, 1934). This fluid's primary constituent is galactogen (Goudsmit and Ashwell, 1965) which provides the main energy source for the developing embryo (Horstmann, 1965; Goudsmit, 1976). Growth and differentiation of the albumen gland in stylommatophoran snails and slugs are under the endocrine control of both the gonad (Abeloos, 1943; Laviolette, 1954; Sokolove *et al,* 1986) and the dorsal bodies (Wijdenes and Runham, 1976). With regard to the synthetic activity of the albumen gland, *in vitro* experiments demonstrated a direct endocrine control by factors produced either in the central nervous system (Goudsmit, 1975, 1978) or in the non-nervous dorsal bodies (Van Minnen and Sokolove, 1984).

In *Helix aspersa*, the direct stimulation of organites implicated in the albumen gland secretion by the gonad was demonstrated using an ultrastructural study of organ associations cultured on a semi-solid medium (Gomot and Courtot, 1979). Castrations and gonadal implantations indicated that in addition to growth and differentiation, galactogen synthesis in the albumen gland is stimulated by implantation of gonads removed from active snails. In addition, it was shown that implantation of gonads from hibernating snails caused an increase of albumen gland glycogen secretion whereas gonads from active snails caused a stimulation of galactogen synthesis (Berset de Vaufleury *et al,* 1986). Therefore, it appears that the physiological state of implanted gonads interferes with experimental results.

The aim of the present *in vitro* study in *H aspersa* was, first, to investigate a liquid medium assay in which the determination of the galactogen synthesis by incorporation of ^{14}C-glucose would be easier than ultrastructural observations. Secondly, this study was undertaken to check whether the gonadal effect demonstrated ultrastructurally in some organites of cultured albumen glands (Gomot and Courtot, 1979) corresponds to the stimulation of the galactogen synthesis and whether variations of this effect are correlated to physiological stages of the sexual cycle. Investigations were made in both virgin and mated snails.

MATERIALS AND METHODS

Animals

One-month-old sexually immature snails were raised individually in 500 ml plastic containers under constant temperature (20°C), photoperiod (18 h L, 6 h D) and relative humidity (95%). They were fed powdered food (UCAAB, Chierry, 02400 Château-Thierry, France) *ad libitum* once a day. These snails attained adult size and became sexually mature at 4 months, coincident with the upturning of the shell edge. Similarly, one-month-old snails were raised in groups of 10 in 2 000 ml plastic containers under the same conditions.

A glass jar filled with moist soil was provided for egg laying. Each snail was numbered with adhesive tape. The reproductive activity (copulations, egg laying) was carefully monitored (once in the morning, once in the evening). This allowed us to know exactly the reproductive stage of each animal. In this way, it was possible to choose pairs of snails to study the effect of either single or repeated mating on the *in vitro* response of the albumen gland galactogen synthesis to the gonadal extracts.

In *H aspersa*, egg laying is generally preceded by multiple mating as it is reported in other species (Van Duivenboden and Ter Maat, 1985; Baur, 1988; Khan *et al,* 1990). Several data demonstrated the importance of mating as a stimulant of the female sexual activity as compared to virgin snails (Van Duivenboden, 1983; Saleuddin *et al,* 1983, 1989; Khan *et al,* 1990; Saleuddin *et al,* 1991).

Tissue preparation

The albumen gland was removed from virgin, single-mated or repeatedly mated snails. After the animal had been weighed, the albumen gland was removed and its maturation index (mi) was calculated:

$$mi = \frac{\text{wt of albumen gland}}{\text{wt of animal}} \times 100$$

Explants of albumen gland were then prepared as previously described (Bride *et al,* 1991). Five samples were used for control conditions and groups of 5 other samples for experimental conditions.

The gonads were cleanly separated, teased away from the underlying hepatopancreas with fine forceps and then rinsed in a saline solution (McCrone and Sokolove, 1979). Each gonad was homogenized in a minimal volume (50 µl) of 0.1 M Hepes buffer, pH 7.4, then centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant contained the gonadal extract of one animal equivalent (1 ae).

For the study of the dose–response relationship, gonads were removed from a population of adult reproductively active *H aspersa* in the dominant male phase after egg-laying. The supernatants were gathered together in a pool (R). Pooled gonads were also removed from 4-month-old virgin snails (V).

In paired snails, gonads and albumen glands were removed within 6 h after copulation and immediately prepared for culture. The stage of differentiation of the gonads was estimated by careful examination under a microscope. In *H aspersa,* the ovotestis first shows a male phase and contains mainly spermatozoa. This stage is gradually replaced by a phase of oogenesis including a primary vitellogenic stage characterized by small oocytes and a secondary vitellogenic stage containing large oocytes.

Each gonad of mated snails was individually homogenized and the supernatant was divided in 2 parts of 0.5 ae. Thus, in each pair, a 0.5 ae of gonadal extract of one of the partners was added to autologous albumen gland explants and the other 0.5 ae to the explants from the second partner and *vice versa* (see fig 2 below).

Culture medium

The liquid medium containing ^{14}C-glucose as precursor for galactogen synthesis was prepared as previously described (Bride *et al,* 1991). The culture time was 24 h at room temperature in the dark.

Determination of the galactogen synthesis

The galactogen synthesis was determined by measuring the incorporation of ^{14}C-glucose as previously described (Bride *et al,* 1991).

Statistical analysis

The results were statistically tested in a one-way analysis of variance followed by the multiple range test of Newman and Keul (Zar, 1978) at a probability level of 0.05.

RESULTS

Experiments in virgin snails

Effect of gonadal extracts on the albumen gland of virgin snails

Five 4.5-month-old virgin snails were randomly chosen. The influence of the pooled gonadal extract of either reproductively active snails (R) or virgin snails (V) was investigated on the *in vitro* incorporation of ^{14}C-glucose in galactogen synthesized by explants of the albumen glands. Galactogen was synthesized by individual albumen glands at different basal rates as reported in Goudsmit (1978) and Van Minnen *et al* (1983). ^{14}C-Glucose incorporation in the presence of R or V did not vary significantly from the control basal synthetic activity (table I). Thus, the albumen glands of 4.5-month-

Table I. Effect of gonadal extracts on galactogen synthesis in albumen glands from virgin snails.

Albumen gland mi	*^{14}C-Glucose incorporation (dpm/mg wet weight; m ± SD)*		
	Control	*R (1 ae)*	*V (1 ae)*
5.48	776 ± 280	585 ± 330	780 ± 170
8.12	1 130 ± 550	1 300 ± 516	1 020 ± 440
9.82	1 092 ± 530	750 ± 490	930 ± 505
10.98	615 ± 90	695 ± 150	640 ± 110
12.18	350 ± 60	280 ± 17	310 ± 40

In vitro glucose incorporation in newly synthesized galactogen in albumen gland explants from virgin snails in the presence of 1ae (animal equivalent, see *Materials and methods*) of gonadal extract from either reproductively active snails (R) in dominant male phase after egg-laying or virgin snails (V). n = 5; mi: maturation index (see *Materials and methods*).

old virgin snails are not responsive to gonadal influence *in vitro*.

Demonstration of the galactogen synthesis stimulatory activity of the gonad of virgin snails

The pooled extract of gonads of virgin snails (V) added to explants of albumen gland from a reproductively active snail in the male phase after egg-laying (mi = 3.3) caused a significant increase of galactogen synthesis by 88% for 1 ae as compared to the control (fig 1).

Experiments in mated snails

Effect of gonads of reciprocally mated snails tested on their own albumen gland synthesis

Because a difference in the level of response of the albumen gland was found between the first-mated and the repeatedly mated snails, in our experiments we selected snails for which copulation occurred for the first time for snail 1 and for the second time without egg-laying for snail 2 (fig 2).

The albumen gland of single-mated snail 1 was characterized by a mi of 3.05 whereas that of twice-mated snail 2 is larger (mi = 8.36). The galactogen synthesis in control medium was also different between the partners. In twice-mated snail 2, the basal synthesis was 53% significantly lower than control 1. Nevertheless, the albumen gland of the repeatedly mated

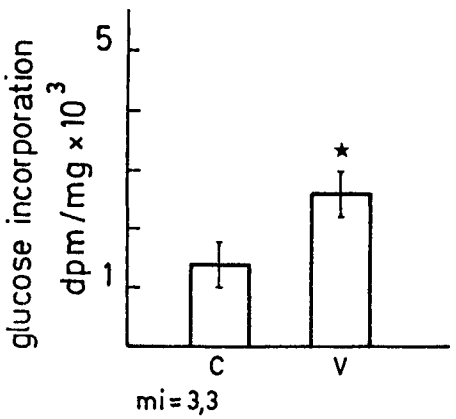

Fig 1. Effect of gonadal extract from virgin snails (V) on galactogen synthesis of the albumen gland from a mature snail after egg-laying. The star indicates a significant stimulation. Mean ± SD (n = 5), $p < 0.05$. C = control (albumen gland without gonadal extract).

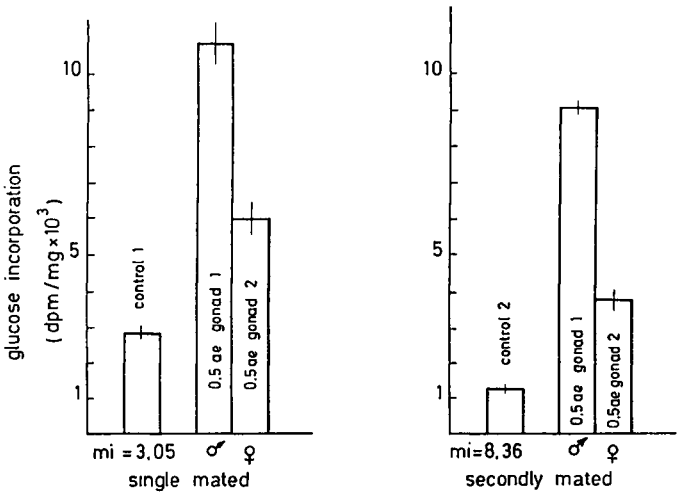

Fig 2. Galactogen synthesis by albumen gland of snails mated once (1) and twice (2). Effect of 0.5 ae of gonadal extract from male (1) or female (2) phase gonads. Mean ± SD ($n = 5$), $p < 0.05$.

snail 2 was more responsive to the 0.5 ae of gonadal extracts than that of single-mated snail 1. Indeed, 0.5 ae of the male phase gonad of snail 1 caused an increase of galactogen synthesis in albumen gland from snail 2 of 610% compared with control 2 and only of 280% in albumen gland from snail 1 as compared with control 1. The gonad from twice mated snail 2 in the first vitellogenic female stage was less active than the male gonad of the single-mated snail 1. The gonad from snail 2 (0.5 ae) increased galactogen synthesis by only 110% compared control 1 and 183% compared with control 2.

In another experiment the pair consisted of snail 3 (which copulated for the first time in the second cycle of matings and egg-laying, after emptying its albumen gland and gonad by egg-laying) and snail 4 which cop-

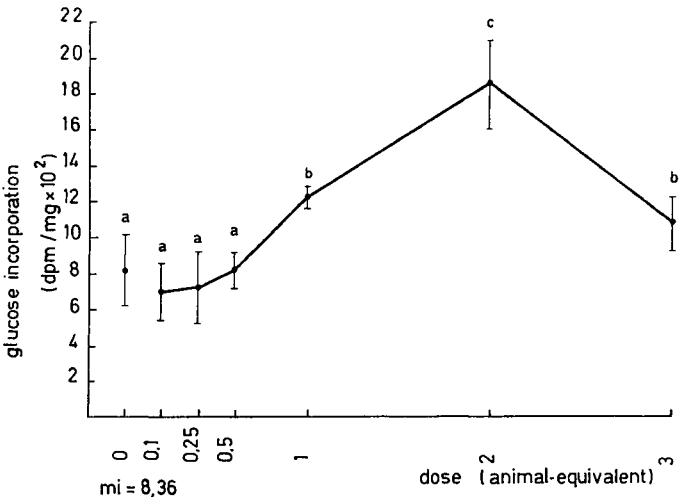

Fig 3. Dose–response relationship of galactogen synthesis stimulation in albumen gland from a mated snail by gonadal pooled saline extract of reproductively active snails in the dominant male phase. Each point represents mean ± SD of 5 explants and those sharing a common letter do not differ significantly ($p < 0.05$).

ulated for the third time in its first cycle without having laid eggs.

Albumen gland mi of the partners were different: 4.65 for the first-mated snail 3 in male phase, and 11.2 for the repeatedly mated snail 4 in second vitellogenic female phase. The basal activity (control 4) of the large albumen gland from snail 4 was significantly 3 times lower ($p < 0.01$) than that from snail 3 (control 3). Only the minute gland from the first-mated snail 3 was stimulated by the 0.5 ae of its male phase gonad (3) which increased galactogen synthesis by 68%. The large albumen gland from snail 4, which was mated 3 times, did not respond to the stimulatory activity of the male gonad from snail 3 (0.5 ae). The extract of the gonad from snail 4, in second vitellogenic stage of the female phase, did not significantly stimulate the sensitive albumen gland from snail 3 or the large albumen gland from snail 4.

Dose–response relationship of the effect of pooled gonadal extract on *in vitro* galactogen synthesis

The influence of aliquots of gonadal extract R on the galactogen synthesis of the albumen gland (mi = 8.6) removed the day after the first mating was demonstrated (fig 3). The minimum dose to elicit a significant stimulation over the control is 1 ae which increased the ^{14}C-glucose incorporation by 50% compared with the control. The dose to obtain a maximum response was 2 ae which caused a rise of galactogen synthesis by 102% compared with the control.

DISCUSSION AND CONCLUSION

A point illustrated by this study is that the gonad of adult active *H aspersa* appears to contain a factor which exerts a direct stimulatory activity on galactogen synthesis of albumen glands in the appropriate stage of the sexual cycle.

The above *in vitro* results do not agree with observations in basommatophorans where castration experiments have indicated that neither the development nor the activity of the accessory sex organs is under endocrine control of the gonad (Brisson, 1971; De Jong-Brink *et al,* 1979; Vianey-Liaud, 1979).

The results demonstrate that the level of *in vitro* stimulation of albumen gland synthetic activity by crude saline gonadal extracts depends on the receptivity of the albumen gland and on the differentiation stage of the gonad. These 2 parameters vary with the reproductive stages of the snails. Three stages of reproductive activity have been considered in *H aspersa*, corresponding to virgin, single-mated and repeatedly mated animals.

Albumen gland receptivity and reproductive stage of the snail

The albumen gland from isolated virgin animal does not respond to the stimulatory activity of the gonadal extract. Two hypothesis may be proposed concerning this observation:

1) The receptors implied in the stimulatory activity of the gonadal extract are not activated, due to the lack of mating.

2) As it was demonstrated in *Helisoma duryi* (Miksys and Saleuddin, 1987), we have observed that the albumen glands of isolated virgin animals are more developed than in reproductive snails. A large amount of secretion stored in the albumen gland may explain the lack of galactogen synthesis stimulation we observed. This has been previously described in *Lymnaea stagnalis* (Wijdenes *et al,* 1981), *Limax maximus* (Van Minnen *et al,* 1983) and *H duryi* (Miksys and Saleuddin, 1985), where a large quantity of secretory material in albumen gland may

inhibit the responses to galactogen synthesis stimulating factors from parts of the brain.

After mating, there is a marked difference in the level of the stimulation observed *in vitro* between the albumen gland from single-mated and repeatedly mated snails. At the second mating, the *in vitro* stimulation of the albumen gland galactogen synthesis is higher than in snails mated either once or three times. The first and the second mating promote the receptivity of the albumen gland for the *in vitro* stimulation of galactogen synthesis by the gonadal extract. It can be extrapolated that the activation of the galactogen synthesis machinery would be caused by the transfer of sperm and male secretions from the partner (Van Duivenboden, 1983), which have a direct stimulatory effect on the albumen gland (Bride and Gomot, 1991b). At the third mating, the large albumen gland becomes unresponsive *in vitro* to the stimulatory activity of the gonadal extract. Moreover, its low basal synthetic activity (control value) suggests that the *in vivo* level of galactogen synthesis has also decreased. The absence of a significant effect of the gonadal extract on the synthetic activity of the large gland indicates that the storage of the secretory product arising from synthesis of galactogen after mating (Bride *et al,* 1991; Bride and Gomot, 1991a,b) may account for the lack of stimulation for further synthesis of galactogen. As observed in *L stagnalis* (Wijdenes *et al,* 1981) and *L maximus* (Van Minnen *et al,* 1983), the quantitative response of the albumen gland of *H aspersa* can change considerably with the weight of the gland, or mi, which varies in different stages of the sexual cycle.

Relation between the stage of differentiation of the gonad and the effect of gonadal extract on galactogen synthesis

The nature of the stimulatory gonadal factor is unknown. However, it appears that *in vitro* galactogen synthesis in albumen glands is more active in the presence of extract of male phase gonad in spermiogenesis (at the first mating) than under the influence of extract of vitellogenic female phase gonad (second and third mating). This difference of influence between predominantly male and predominantly female gonads was also demonstrated in the *in vitro* study of polysaccharide synthesis in the oviduct of *H aspersa* (Bride and Gomot, 1988). The reason for these differences is not known. Nevertheless, they can be correlated with previous studies on implantation with male gonads in *H aspersa* (Berset de Vaufleury *et al,* 1986) and in slugs (Laviolette, 1954) where implantation of gonads at the end of the spermiogenesis caused albumen gland and common duct enlargement whereas there was no response to implantation of gonads that had not begun spermiogenesis. In virgin *H aspersa*, the gonads which elicit galactogen synthesis stimulating activity are full of spermatozoa and contain only previtellogenic oocytes. This was also observed in *H duryi*, whose vitellogenesis was arrested in virgins because of the inactivity of the dorsal bodies (Saleuddin *et al,* 1983).

In conclusion, these results indicate the possibility of using this albumen gland bioassay to test the effect of fractions isolated by chemical extraction of the gonad. It also raises questions related to the cause of the evolution of albumen gland receptivity with respect to the role of repeated matings preceding egg-laying and the existence of a cycle in the production of gonadal endocrine factor(s) with respect to the correlation between: a) spermiogenesis and the presence of a gonadal galactogen synthesis stimulatory substance; and b) vitellogenesis and the disappearance of this galactogen synthesis stimulatory substance.

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