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ROLES OF NEUROSECRETORY CELLS ON GROWTH AND REPRODUCTION AND THEIR SEASONAL VARIATION IN *ACHATINA FULICA* (GASTROPODA : ACHATINIDAE)

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ABSTRACT

The injections of Achatina fulica *with cerebral ganglion homogenate and with optic tentacle homogenate revealed that both substances had no effect on body growth of* A. fulica. *However, the cerebral ganglion homogenate appeared to increase the oocyte production in the ovotestis of snails. Seasonal variation in number and size of neurosecretory cells in the cerebral ganglia of* A. fulica *was observed. The number of neurosecretory cells in the cerebral ganglia was high during the rainy season (May-October,1993) and low during the dry season (November - April, 1993). The highest number of neurosecretory cells and the largest cell size were observed in May. The number and size of collar cells in the optic tentacles were similar throughout the year.*

INTRODUCTION

The occurrence of neurosecretory cells had been investigated in many species of pulmonate snails, i.e., *Lymnaea stagnalis*[1,2], *Australorbis glabratus*[3], *Helisoma tenue*[4], *Helisoma duryi*[5], *Arion ater*[6] and *Achatina fulica*[7]. Various types of neurosecretory cells could be distinguished on the basis of their stainability to classical staining methods using chrome-hematoxylin phloxine[8] and paraldehyde-fuchsin.[9]

Neurosecretory cells are characterized by having oval shaped cell with eccentric round nucleus and a single large vacuole. In addition, there are numerous elementary granules present in the cytoplasm. These granules gave a positive reaction to chrome-hematoxylin phloxine and paraldehyde-fuchsin and can often be further differentiated histochemically with alcian blue-alcian yellow[10]. In *A. fulica*, the neurosecretory cells are located at the periphery of the procerebrum of the cerebral ganglion[7].

In various species of stylommatophorans, it was reported that there were cells which gave a strong reaction with these basic neurosecretory cell stains[6,7,11-13]. These neurosecretory cells so called collar cells arranged themselves around the finger-like processes of the tentacular ganglion of *A. ater*[6], *A. fulica*[7], *Helix aspersa*[11], *Limax maximus*[12] and *Archachatina marginata*[13]. These cells are characterized by the presence of round nucleus and granulated cytoplasm with elementary granules.

There are many reports on the functions of neurosecretory cells. The light green

cells in the cerebral ganglia of *L. stagnalis* and the medial cells in the cerebral ganglia of *Agriolimax reticulatus* have a factor that stimulates growth rates of these snails[14,15]. The caudo-dorsal cells in *L. stagnalis* have the effect on egg-laying behavior and ovulation[16]. Further observations on the neurosecretory cells in the pleural, parietal and visceral ganglia of *L. stagnalis* revealed that these cells could be involved in osmoregulation[17-19]. In *H. aspersa*, the cerebral ganglion extract could stimulate the amoeboid movement of oocytes[20].

The experimental study on hormonal control of reproduction in pulmonate slugs had been performed on *Arion subfuscus* and *A. ater*[12]. After removal of optic tentacles, the slugs showed an increase in the number of eggs in comparison with that of the controls. The injections of brain homogenate into the body cavities of these slugs resulted in the number of eggs exceeded that of the control. In contrast, the optic tentacle homogenate injection did not affect the number of eggs in these slugs[12].

Takeda[21] studied the effect of optic tentacles and cerebral ganglia on the number of eggs in *A. reticulatus* and *L. flavus*. He found that the numbers of eggs oviposited after injection with cerebral ganglion homogenate in *A. reticulatus* and *L. flavus* were larger than those oviposited after injection with optic tentacle homogenate and the controls. From these experiments, Takeda[21] concluded that the cerebral ganglion had a factor that stimulated oogenesis while the optic tentacles had a factor that inhibited oogenesis. In addition, Takeda[22] demonstrated specifically that after the removal of the tentacles, including collar cells in many species of slugs, especially *L. flavus*, no spermatozoa were found in the acini of the ovotestis. After injection of the collar cell homogenate, spermatogenesis could be observed. Takeda[22] also reported the direct stimulating effect of the collar cell homogenate on spermatogenesis by using organ culture technique. Therefore, it was concluded that collar cell homogenate acted directly on spermatogenesis as a gonadotropic hormone.[21,22]

The investigation on differentiation of sex cells in the ovotestis has been carried out with the organ culture technique. In *H. aspersa*, the addition of a cerebral ganglion to the culture medium could result in the differentiation of female sex cells[23]. However, the presence of an optic tentacle in the culture medium resulted in the suppression of differentiation of the female sex cells in the ovotestis of *H. aspersa.*[23]

The work on extirpation of neurosecretory light green cells of *L. stagnalis* showed a significant retardation of body growth in the snails[14]. The reimplantation of cerebral ganglia containing light green cells could restore normal growth in these snails. In contrast, the neurosecretory bright green cells had no effect on body growth[24]. Similar effect on the control of body growth was also found in *A. reticulatus*[15]. The neurosecretory medial cells had been shown to stimulate the body growth of *A. reticulatus.*[15]

There are only a few reports on the effect of seasonal variation of neurosecretory cells and their secretion on maturation of the pulmonate reproductive organs. The significant decrease of neurosecretory cells in the cerebral ganglia in June and July was reported in *H. tenue*[4]. The relationship between neurosecretion in the neurosecretory

cells and the season was investigated in *A. ater*[6]. The seasonal variation of the neurosecretory cells was found to be related with the maturation of the reproductive organs of this snail. In *A. fulica*, seasonal variation was found in the production of oocytes in the ovotestis. The number of oocytes was highest during the rainy season (May-August) and lowest during the dry season (January - April)[25].

Most of the reports provided the information relating to the endocrinological control of the cerebral ganglia and optic tentacles on growth and reproduction in the freshwater snails or slugs but virtually none on *A. fulica*. Therefore, one of the aims of the present study is to investigate the effects of the cerebral ganglia and optic tentacles on growth and reproduction of *A. fulica*. In addition, the seasonal variation of neurosecretory cells in both cerebral ganglia and optic tentacles and their relation to reproduction will also be investigated.

MATERIALS AND METHODS

Study on roles of neurosecretory cells on growth and reproduction

The adult *A. fulica* were collected from the field during the rainy season. These snails were anesthetized with nembutal for 30 minutes and the cerebral ganglia and the optic tentacles were dissected out. The connective tissue surrounding the ganglia was removed. Each ganglionic specimen was then cut into small pieces and ground with 0.1 N saline (pH 8.5) by a hand homogenizer tube[12]. The solution was then centrifuged at 3,000 revolutions per minute for 20 minutes. The clear supernatant was collected and subsequently lyophilized by a Leybold-Heraeus lyophilizer. Finally, the powder of either cerebral ganglion or optic tentacle homogenate was left which was then kept in a refrigerator (at 4°C) until use.

A. fulica of four months old were obtained from the culture laboratory of the Center for Applied Malacology and Entomology, Department of Biology, Mahidol University. These experimental snails were divided into four groups as follows: group I : normal control; group II : normal saline injected group; group III : cerebral ganglion homogenate injected group; group IV : optic tentacle homogenate injected group. Ten snails were used in each group, and four replications were performed for each group.

All the snails were kept in the plastic container at 25°C under 12L:12D photoperiod. The diet was supplied ad libitum. The weights of each group of snails were recorded every week throughout the experiment.

The snails in group I were kept under normal laboratory condition without any treatment while those in group II received an intramuscular injection of 10 µl of normal saline. The cerebral ganglion homogenate as well as optic tentacle homogenate was given to the experimental snails, groups III and IV, respectively, in the same manner as in group II with the dose of 125 µg/g body weight.

The snails received three injections at the time interval of seven days. Two weeks after the third injection, the experimental snails were sacrificed and fixed in Bouin's solution, embedded in paraffin, sectioned at 4-5 µm in thickness and stained

with hematoxylin and eosin. Serial sections of the ovotestis from each experimental group were examined under the light microscope. The counting of oocytes with the nuclei present from the serial sections was used as a parameter for oogenesis activity. The results were expressed as the mean number of oocytes per ovotestis for each treatment. The method of ANOVA (analysis of variance) was used to evaluate the significant difference of oocyte number either at the level of 0.05 and 0.01 of probability between the control and treated groups.

Study on the seasonal variation of neurosecretory cells

Approximately 6-10 snails, *A. fulica* of 60-70 mm in shell length, were collected from the field during the first ten days of each month from January to December 1993. Each snail was measured, and the cerebral ganglia and optic tentacles were dissected out. They were fixed in Bouin's solution, embedded in paraffin and sectioned at 4-5 μm in thickness and stained with chrome-hematoxylin phloxine[8] and paraldehyde fuchsin[9].

The neurosecretory cells with nuclei present were counted from the whole sections by using a multi-square graticule in the eyepiece. The result was expressed as the mean number of neurosecretory cells per ganglion and per mm^2 for cerebral ganglion and optic tentacle, respectively. The size of the cells was measured by using ocular grid. The variations in numbers of neurosecretory cells per cerebral ganglion and collar cells per mm^2 in optic tentacles were analyzed by the method of ANOVA, and the mean diameters of neurosecretory cells in cerebral ganglia and optic tentacles were compared by the method of t-test.

RESULTS

Effects of administration of cerebral ganglion and optic tentacle homogenates on body growth

The initial average weights of the four groups of *A. fulica* in the first week of the experiment are 10.2±0.4 g in control group, 11.7±0.6 g in normal saline (NS) injected group, 12.3±0.5 g in cerebral ganglion homogenate (CGH) injected group and 11.3± 0.3 g in optic tentacle homogenate (OpTH) injected group (Fig.1). On the second week, after the first injections, the mean weights of the experimental snails are increased to 14.4±0.6, 13.7±0.5 and 14.7±0.6 g, respectively whereas the mean weight of the control is 13.7±0.6 g. The experimental snail weights, however, do not show any significant difference from the control group.

On the third week, after the second injections, the average weights of the experimental groups (CGH and OpTH) were still increased (Fig.1). No significant changes are observed when compared these two treatments (CGH and OpTH) with both the control and NS injected groups. The mean weights are 16.4±0.06 and 17.7 ±0.7 g in CGH and OpTH injected groups respectively, whereas the weights of the control and NS injected groups are 18.9±1.2 and 17.3±0.9 g, respectively.

After the third injections of CGH and OpTH, the increase in weight of these

snails is still observed. During the fourth and fifth weeks, the mean weights of the CGH injected snails are 18.9±0.8 and 22.2±1.3 g, respectively while in the OpTH treated group, the mean weights are 20.9±1.1 and 22.8±1.0 g (Fig.1). The average weights of the control group are 22.4 ±1.0 and 24.7±1.3 g while those of the NS injected group are 20.7±1.1 and 21.9±0.8 g (Fig.1). Statistically, the increases in weights of the experimental groups (CGH and OpTH) are not significantly different from those of the control or the NS injected groups (Fig.1).

Effects of administration of cerebral ganglion and optic tentacle homogenates on oocyte development

The counting of oocytes in the control group (80.7±12.24 oocytes/ovotestis) does not differ statistically from the NS injected group (95.7±9.7) (Fig.2). The result of the oocyte count in the CGH injected group is 173.9±16.76 oocytes/ovotestis (Fig.2). Thus, there is a remarkable increase in the number of oocytes after the injection of CGH. The mean differences (93.2 and 78.2 oocytes/ovotestis) are statistically significant ($p < 0.01$).

The oocyte count of the OpTH injected group (87.6±13.23 oocytes/ovotestis) reveals that there is a slight increase in the number of oocytes as compared to that of the control group (Fig.2). The mean difference (6.9 oocytes/ovotestis) is not statistically significant ($p > 0.01$).

The comparison between the OpTH injected and NS injected groups shows that there is a slight reduction in the number of oocytes in the OpTH group as compared to that in the NS group (Fig.2). The mean difference (8.1 oocytes/ovotestis) is not statistically significant ($p > 0.01$).

Seasonal variation of neurosecretory cells in the cerebral ganglia and optic tentacles

The average number of neurosecretory cells in the cerebral ganglia of *A. fulica* is significantly different in certain months of the year ($p < 0.05$) (Fig.3). The number of neurosecretory cells from January (49.3 cells per cerebral ganglion) to February (42.7 cells per cerebral ganglion) is not significantly different. The number of neurosecretory cells increases from March (57.0 cells per cerebral ganglion) to April (127.3 cells per cerebral ganglion) and rises to a peak in May (187.7 cells per cerebral ganglion), then decreases in June (148.0 cells per cerebral ganglion) and July (135.5 cells per cerebral ganglion), recovers in August (120 cells per cerebral ganglion) and declines again from September to December (73.0, 50.7, 58.0 and 45.0 cells per cerebral ganglion, respectively).

The sizes of neurosecretory cells in the cerebral ganglia from January to December are shown in Fig.4. From January to April, the sizes of neurosecretory cells are the same (Figs. 5A,5B,5C). The cells contain numerous small vacuoles (Fig.5C). The neurosecretory cells steadily increase in size from April to May and become largest in May (Fig.6A). The cells contain a single large vacuole. They are reduced in size in June and July (Fig.6B). The neurosecretory cells continue to reduce in size from August to December (Fig.6C).

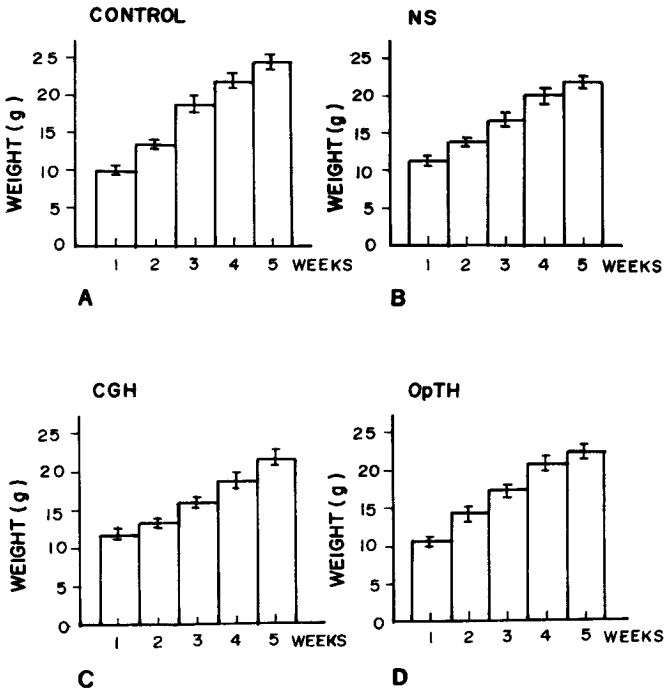

Fig. 1. Histograms showing the weights of *A. fulica* in control group (A), normal saline (NS) injected group (B), cerebral ganglion homogenate (CGH) injected group (C) and optic tentacle homogenate (OpTH) injected group (D).

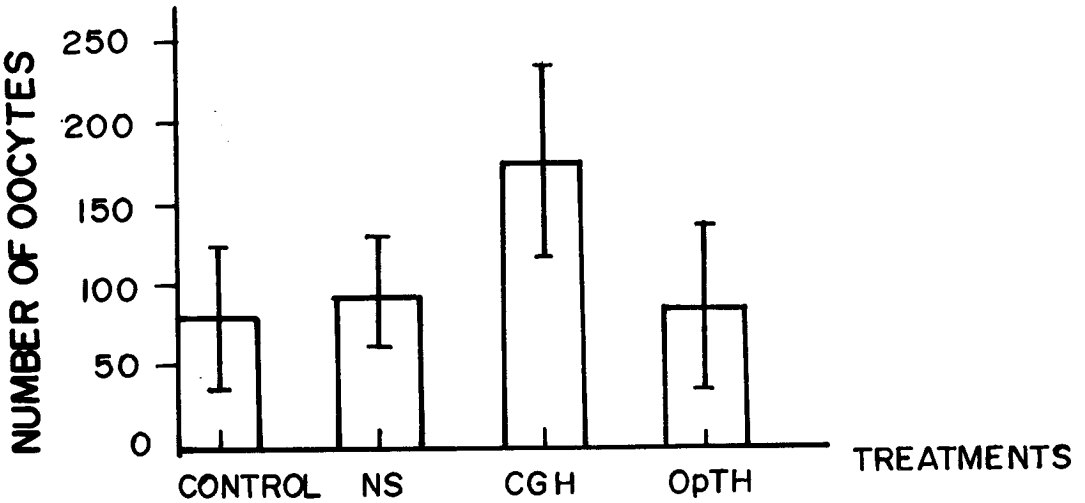

Fig. 2. Histograms showing the numbers of oocytes in control group, normal saline (NS) injected group, cerebral ganglion homogenate (CGH) injected group and optic tentacle homogenate (OpTH) injected group.

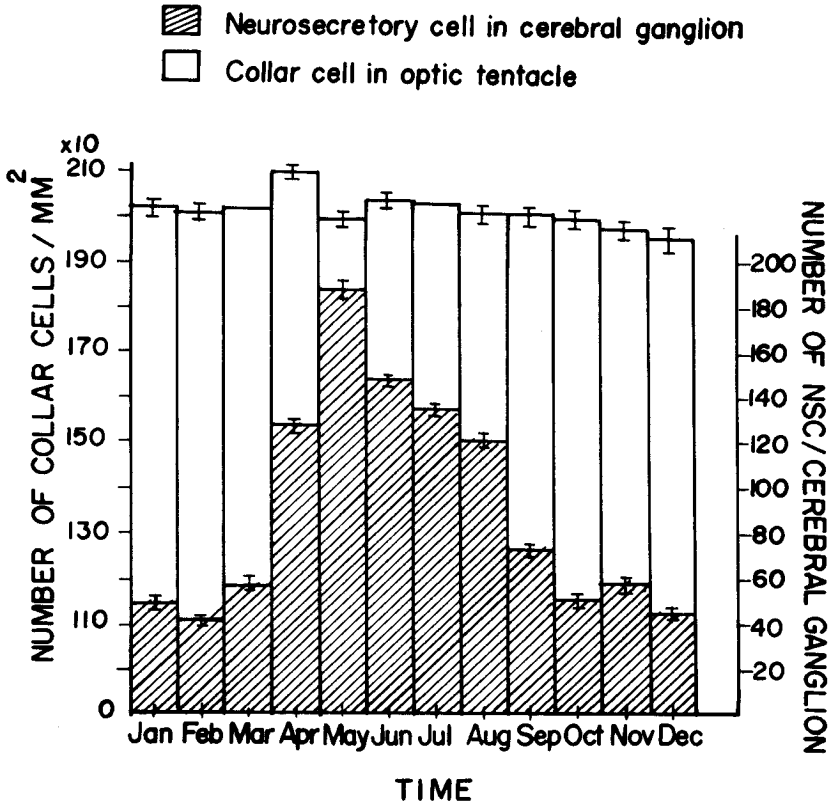

Fig. 3. Histograms showing the numbers of neurosecretory cells (NSC) in the cerebral ganglia and collar cells per mm^2 in the optic tentacles of *A. fulica* in various months throughout the year (1993). Significant difference at $p<0.05$.

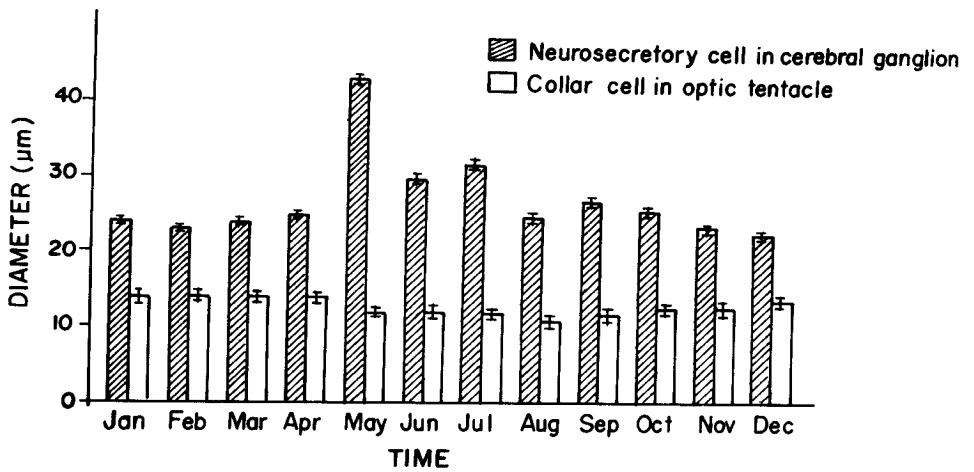

Fig. 4. Histograms showing the sizes of neurosecretory cells in the cerebral ganglia and collar cells in the optic tentacles of *A. fulica* in various months throughout the year (1993).

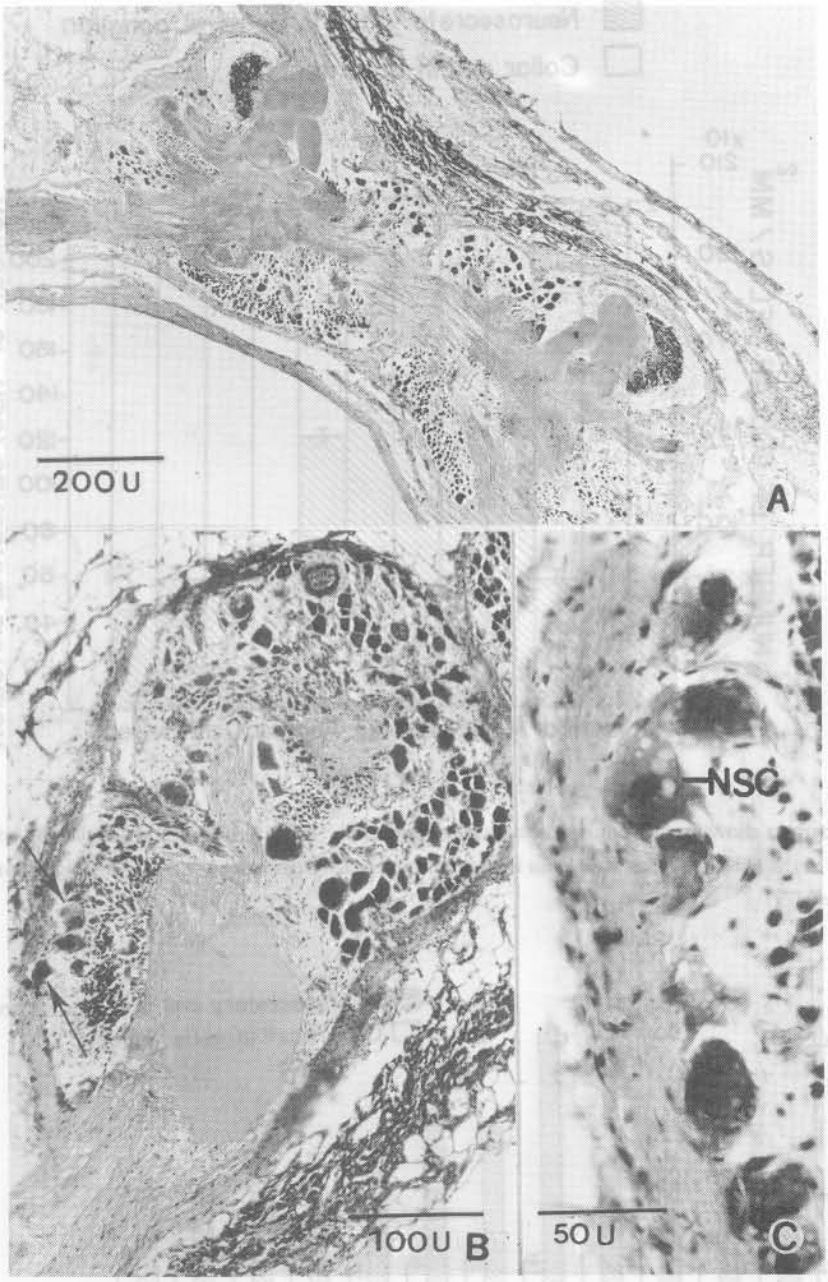

Fig. 5. A. Low magnification of cerebral ganglia of snails obtained in January.

B. Higher magnification of cerebral ganglia of snails obtained in January showing neurosecretory cells (arrows) which are arranged in 2-3 layers in the procerebrum.

C. Neurosecretory cells (NSC) in the cerebral ganglia of snails obtained in January through April. The cells are small in size and contain numerous small vacuoles.

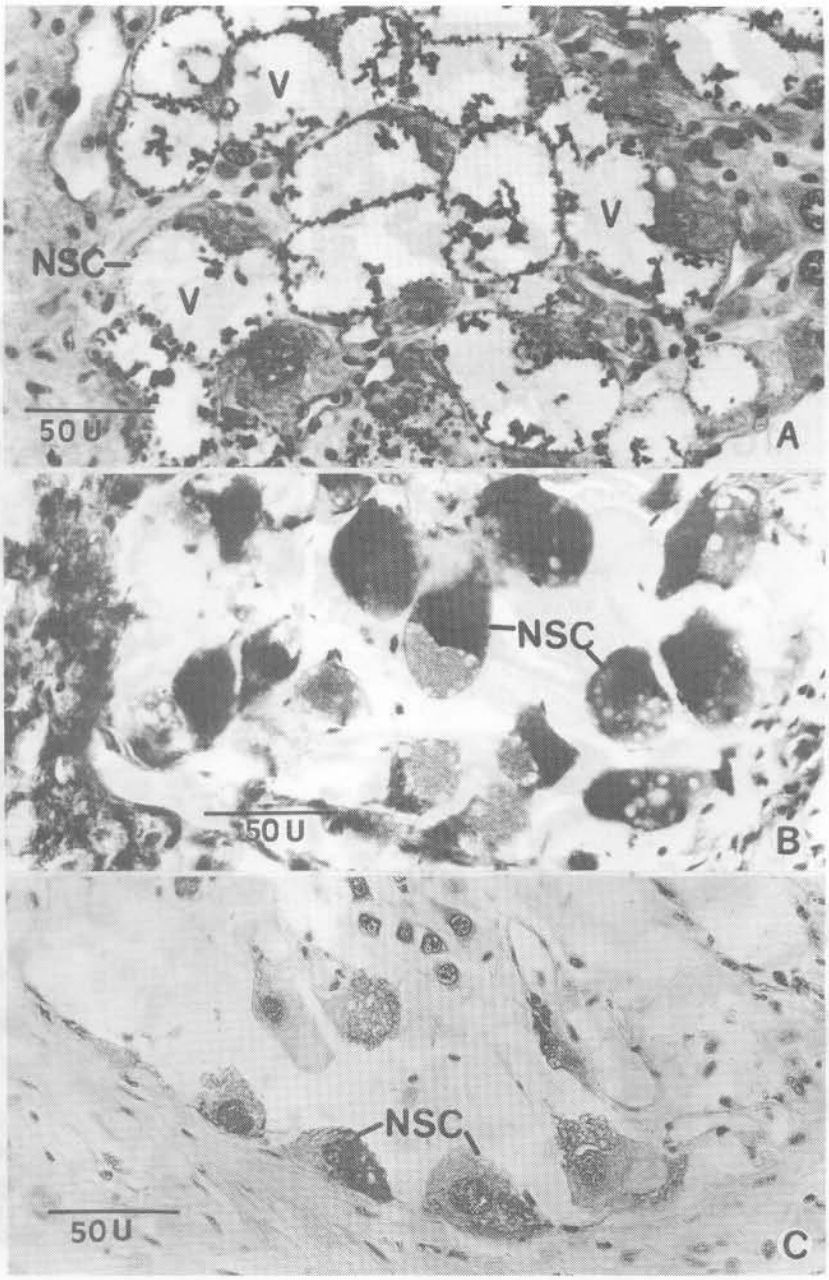

Fig.6. **A.** Neurosecretory cells (NSC) in the cerebral ganglia of snails obtained in May. The cells are largest in size and contain a single large vacuole (V).

B. Neurosecretory cells (NSC) in the cerebral ganglia of snails obtained in June through August. The cells are reduced in size and contain small vacuoles.

C. Neurosecretory cells (NSC) in the cerebral ganglia of snails obtained in September through December. The cells are reduced in number but are of similar size to those in June through August.

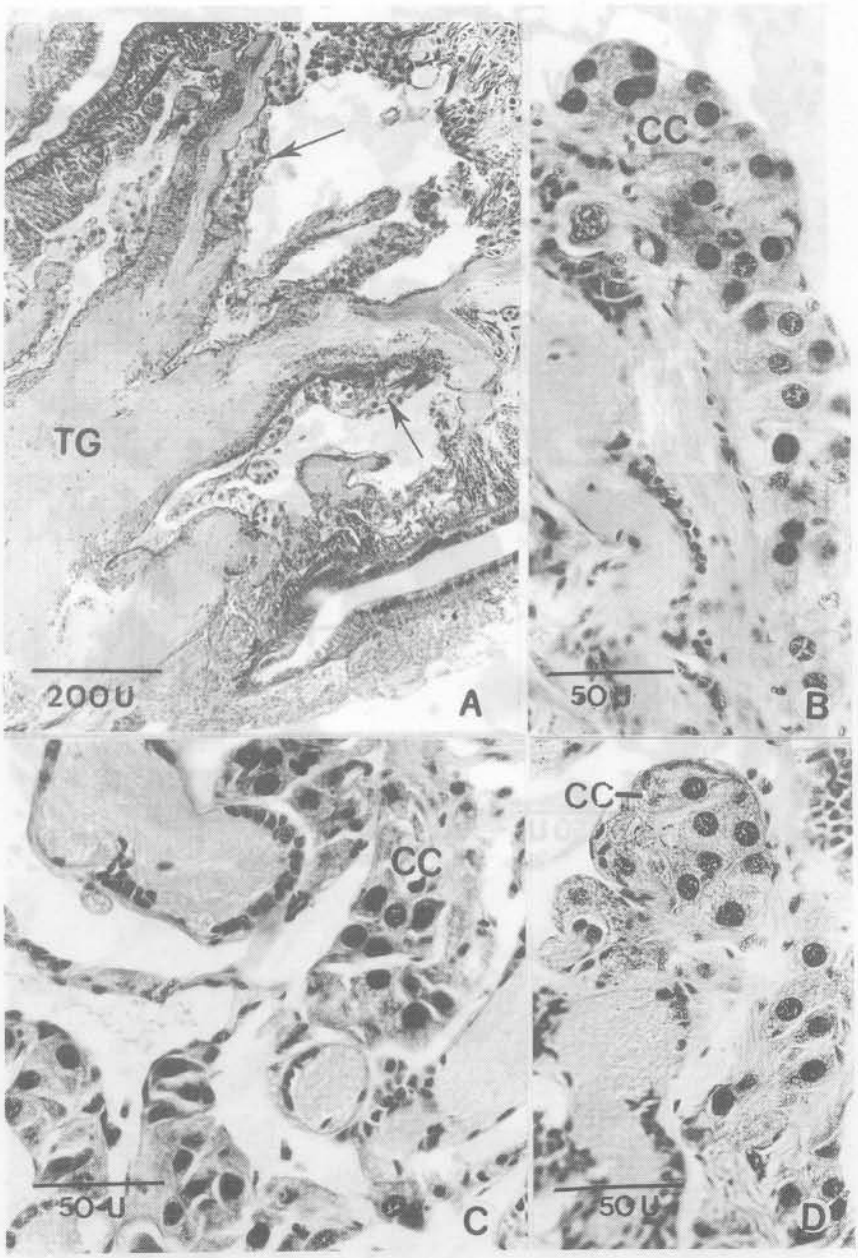

Fig.7. **A.** Low magnification showing tentacular ganglion (TG) with finger-like projections in the optic tentacles. Note the presence of collar cells (arrows) around the tentacular ganglion.

B. Groups of collar cells (CC) in the optic tentacles of snails obtained in January through April.

C. Groups of collar cells (CC) in the optic tentacles of snails obtained in May.

D. Groups of collar cells (CC) in the optic tentacles of snails obtained in June through December.

The average numbers of collar cells in the optic tentacles of *A. fulica* in each month of the year are shown in Fig.3. From January to March, the numbers of collar cells (2018, 2011 and 2022 cells per mm^2) are not significantly different ($p > 0.05$). In May, the number of collar cells (2000 cells per mm^2) is lower than those of the previous months, then recovers in June (2044 cells per mm^2 respectively) and July (2030 cells per mm^2), declines again from August to November (2010, 2008, 2000 and 1980 cells per mm^2, respectively) and decreases in December (1955 cells per mm^2).

The sizes of the collar cells in the optic tentacles are shown in Fig.4. The collar cells are of the same size from January to April (Figs.7A,7B). They become reduced in size from May through August (Fig.7C), then increase again in September to December (Fig.7D).

DISCUSSION

Effects of administration of cerebral ganglion and optic tentacle homogenates on body growth

The present study reveals that the cerebral ganglion and optic tentacle homogenates did not affect body growth of snails, *A. fulica* since the differences in the gain of body weight of the experimental and control snails did not reach statistical significance. This finding does not agree with that of Geraerts[24] that the removal of light green cells in the cerebral ganglia of *L. stagnalis* could stop the growth of the body. This similar effect was also reported in another species of snails, *A. reticulatus*[15]. The different result is probably due to the differences in the anatomy of these experimental snails. However, it was reported that the administration of optic tentacle homogenate resulted in a reduction of growth rate of both young and adult snails, *H. aspersa*[26].

Effects of administration of cerebral ganglion and optic tentacle homogenates on oocyte development

The study on ovotestis of *A. fulica* after the administration of cerebral ganglion homogenate showed the presence of numerous oocytes in most acini of the ovotestis. These similar results were reported in other snails, i.e., *Arion*[12] and *Milax*[27]. The ovotestis of *Milax* showed a marked increase in the number of oocytes, particularly after the injection of brain hormone rather than tentacular hormone[27].

In addition, there was an evidence which showed that neurosecretory cells in the cerebral ganglia of *L. stagnalis* played a particular role in the process of oogenesis and oviposition[28]. The present study also showed that the administration of cerebral ganglion homogenate to *A. fulica* could lead to a stimulation of female phase in the ovotestis. Ram[29] reported that the extracts of nervous system caused the laying of egg capsules in marine prosobranch, *Busycon*. Furthermore, Gomot and Gomot[30] reported that hormone from the brain of *H. aspersa* inhibited multiplication of spermatogonia and spermatocytes. In *A. fulica*, the oocytes were increased and more developed than the spermatocytes in the cerebral ganglion homogenate injected snails. Hence, the

increase in the number of oocytes in the ovotestis of the cerebral ganglion homogenate injected group might be stimulated by a hormone from the cerebral ganglia.

The effects of optic tentacle homogenate on ovotestis had been studied in many species of snails. In *A. subfuscus* and *A. ater*, the neurosecretory cells in the optic tentacles were reported to control sperm formation and at the same time could suppress differentiation of the eggs[12]. Takeda[21,22] who studied on slug, *L. flavus*, had confirmed that the optic tentacles had direct effect on spermatogenesis. The present study shows that in *A. fulica*, the average number of oocytes in the optic tentacle homogenate injected group is roughly the same as those in the control and normal saline injected groups. This finding might suggest that the excess amount of optic tentacle homogenate given to *A. fulica* could not cause any inhibiting effect on the female phase differentiation as occurred in *Arion* spp.

Seasonal variation of neurosecretory cells in the cerebral ganglia and optic tentacles

Seasonal variation in number and size of neurosecretory cells in the cerebral ganglia of *A. fulica* was observed. The number of neurosecretory cells was high during the rainy season (May-October, 1993) and low during the dry season (November-April, 1993). However, the number and size of collar cells in the optic tentacles were similar throughout the year. The correlation between the number of neurosecretory cells in the cerebral ganglia and the seasonal variation can also be found in *H. tenue*[4], in which the number of these cells was highest in June and lowest in March, whereas the number of neurosecretory cells in the cerebral ganglia of *A. fulica* was highest in May and lowest in February. The differences in both snails may be due to their anatomy and habitats, i.e., *H. tenue* is a freshwater basommatophoran snail, while *A. fulica* is a land stylommatophoran snail.

There seems to be a correlation between the variation in the number and size of neurosecretory cells in the cerebral ganglia and the production of oocytes in the ovotestis. In the present study, the highest number of neurosecretory cells and the largest cell size were observed in May. The study on seasonal variation in the ovotestis of *A. fulica* revealed that the number of oocytes in the ovotestis was also highest in May[25]. Hence, it is suggested that the neurosecretory cells in the cerebral ganglia produce neurohormones which can stimulate the oocyte production.

Seasonal variation such as photoperiod and rainfall, may have indirect effects on the maturation of the reproductive organs. *A. fulica* in Malaysia had intense reproductive activity from July to December and declined to a minimum in March[31]. It was also observed that the reproductive cycle of *A. fulica* was related to rainfall, and that the differences in the amount of rainfall affected the fluctuations in reproductive condition[31].

Photoperiod is another factor that has been suggested to have the effect on the activity of neurosecretory cells in the cerebral ganglia and the maturation of the reproductive organs. The long-day period in the summer is the essential factor which stimulates the activity of neurosecretory cells in the cerebral ganglia and has the indirect effect on the maturation of reproductive organs in *L. stagnalis*[28,32]. Bohlken and Joosse[32] found that the long-day *L. stagnalis* started oviposition seven weeks after hatching,

which was two weeks earlier than the snails maintained in the medium day and short day. Moreover, the long-day snails showed a rapid increase in egg production 5.6 and 9.5 times higher than those of the medium-day and short-day snails, respectively[32].

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บทคัดย่อ

ผลของการทดลองโดยการฉีดสารสกัดจากปมประสาทเซรีบรัลและออปติกเทนเทเคิลที่มีเซลล์นิวโรเซครีทอรีเข้าไปในหอยทากยักษ์ *Achatina fulica* พบว่า สารสกัดทั้งสองนี้ไม่มีผลต่ออัตราการเจริญเติบโตของหอยทากยักษ์ แต่สารสกัดจากปมประสาทเซรีบรัลสามารถกระตุ้นการสร้างไข่ในอวัยวะสืบพันธุ์ร่วมของหอยได้

การศึกษาการเปลี่ยนแปลงตามฤดูกาลในจำนวนและขนาดของเซลล์นิวโรเซครีทอรีในปมประสาทเซรีบรัลและออปติกเทนเทเคิล พบว่าในปมประสาทเซรีบรัล เซลล์นิวโรเซครีทอรีมีจำนวนมากที่สุดในระหว่างฤดูฝน (พฤษภาคม - ตุลาคม 2536) และมีจำนวนลดลงในระหว่างฤดูแล้ง (พฤศจิกายน - เมษายน 2536) โดยในเดือนพฤษภาคมจะพบเซลล์นิวโรเซครีทอรีที่มีขนาดใหญ่ที่สุดและมีจำนวนมากที่สุด ส่วนในออปติกเทนเทเคิลนั้น เซลล์นิวโรเซครีทอรีหรือเซลล์คอลลาร์ไม่มีการเปลี่ยนแปลงทั้งขนาดและจำนวนตลอดปี