

HISTOLOGICAL AND HISTOCHEMICAL OBSERVATIONS ON THE REPRODUCTIVE ORGANS OF *Archachatina marginata ovum* (GASTROPODA: ACHATINIDAE) AT DIFFERENT REPRODUCTIVE STATES

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ABSTRACT

The histological and histochemical variation in association with morphological variation in the reproductive system of *Archachatina marginata ovum* was the target of this investigation. Forty-five snails were dissected and categorized into 5 different reproductive stages (low mating, high mating, high egg, gravid and post reproductive). The reproductive tracts which include: hermaphroditic duct, albumen gland, spermooviduct and spermatheca and the ovotestis were processed for histological and histochemical staining. There were some variations in the architecture of the reproductive organs between the active (high mating, high egg and gravid) and non active stages (low mating and post reproductive) states. The active states were generally associated with colloidal or granular secretions. Glycogen and alkaline phosphatase activities were associated together throughout the epithelium of the reproductive system of *A. marginata ovum* and they were more strongly indicated in tissues that are intimately connected to the growth and development of gametes. It was concluded that morphological variation in the secreting glands of the reproductive system of *A. marginata ovum* is closely associated with changes in the functional secretory activities of the glands.

Keywords: *Archachatina marginata ovum*, reproductive system, histochemistry, histology.

INTRODUCTION

Members of achatinidae family are important both as food and pest. Thus, their reproductive system has been the target of investigation for many decades. The works of Mead (1950), Ghose (1963) and Breckenridge and Fallil (1973) are outstanding in understanding the morphology and histology of the different parts of the reproduc-

tive system of *Achatina fulica* and thus provided a solid background for further understanding of the reproductive processes in the achatinidae family. Ngowsiri *et al.* (1989) studied the developmental stages of the reproductive system of *A. fulica* and observed that the ovotestis first appeared in the 3 month old snail with active production of spermatozoa whereas oocytes were produced

later at 5 month of age. They further noted that while spermatozoa were produced continuously throughout the year, oocytes production was restricted to some periods of the year. In *Archachatina marginata ovum*, Egonmwan (2007a) also reported a season-dependent variation in the morphology of the reproductive organs, most importantly the albumen gland. A subsequent study using electron microscopy revealed that the secretory cells of the albumen gland secrete both small droplets and large globules which were believed to be glycogen, particularly during the active reproductive stages (Egonmwan, 2007b). Some histochemical studies of the reproductive systems of few members of the pulmonates gastropod have been reported (Kugler, 1965; Smith, 1965; Benita *et al.*, 1970; Ramasubramaniam, 1979), which showed glycogen and alkaline phosphatase to be well expressed in the reproductive tissues. However, detailed understanding of the achatinidae reproductive functioning is still lacking. In particular, there is little knowledge on the reproductive physiology of the *Archachatina marginata* which is primarily confined to the West African region. Thus, this study examined the histology and histochemistry of the reproductive organs of *A. marginata ovum* at the five different reproductive stages (low mating; high mating; high egg, gravid and post reproductive) described by Egonmwan (2007a).

MATERIALS AND METHODS

The study was conducted in the Animal Reproduction laboratory of Department of Animal Sciences and Histochemistry laboratory of the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, Nigeria. Adult *A. marginata ovum* weighing between 150 – 400 g were bought from wild snail collectors in the raining

month of April and brought to the laboratory where they were dissected within 24 hours to retrieve the reproductive system. Forty-five (45) snails were categorized after dissection into one of the 5 reproductive states according to Egonmwan (2007a) description. Among the snails were 10, 8, 10, 6 and 11 in the low mating, high mating, high egg, gravid and post reproductive states, respectively. After dissection the albumen gland, ovotestis, hermaphroditic duct, spermooviduct and spermatheca were processed for both histological and histochemical staining. Plate 1 show the entire reproductive system of dissected out of the snail and the organs sectioned for staining.

Histological preparations

Each reproductive organ of the snails was fixed in Bouin's fluid for at least 24 hours and later trimmed to about 3-5 mm thickness. The fixed tissues were dehydrated at room temperature through ascending grades of ethanol. Dehydrated tissues were cleared in two changes of xylene for one hour in each change at room temperature. The tissues were then infiltrated in two changes of molten paraffin wax at 60 °C for an hour in each change and finally embedded in paraffin wax using multi-block plastic embedding moulds. Sections of 5-6 µm thickness were produced from the tissue blocks using a rotary microtome (Bright B5143, Bright Instrument, England). The sections were transferred into water bath (40 °C) from where good sections were selected and mounted on clean glass slides, dried at 40 °C on a slide drier and then stained by the procedures of Harris acid haematoxylin-eosin (Sheehan and Hrapchak, 1980).

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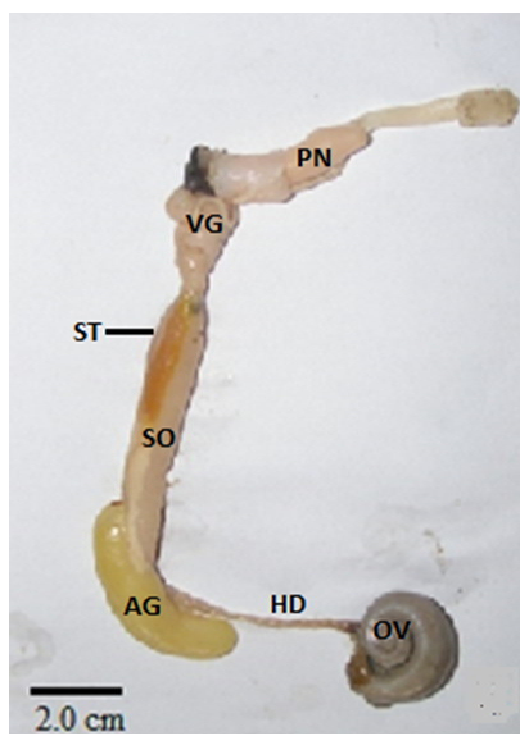


Plate 1: The reproductive system of *A. marginata ovum* at high mating state. AG, albumen gland; HD, hermaphroditic duct; SO, spermoviduct; PN, penis; ST, spermatheca; VG, vagina

Histochemical preparations

Fresh tissues were fixed in 10 % normal saline and later embedded in ice on cryostat (Clinicut 60, Bright Instrument, England) from where frozen sections of 20 µm thickness were cut. Sections were stained by the Best carmine method for the identification

of glycogen, bromophenol blue method for identification of general protein group, Gomori calcium method for the identification of alkaline phosphatase and mucihematin reaction for the identification of mucin. All procedures were as described by Pearse (1960).

RESULTS

Histological Observations

Ovotestis: In the ovotestis of snails in the low mating, high mating and post reproductive states the acini contained only the male gamete, seen at the different stages of spermatogenesis whereas in the high egg and gravid states the acini were primarily composed of oocytes with few spermatids (Plate 2).

Hermaphroditic duct: The hermaphroditic

duct sectioned at the bulbous region considered to be seminal vesicle region (Breckenridge and Fallil, 1973) showed a wide lumen rounded by columnar epithelium and connective tissue layer. The lumen contained large number of spermatozoa in all the reproductive states. However in the high egg reproductive state large quantity of brown granular deposit was also observed in the cytoplasm of the epidermis (Plate 3).

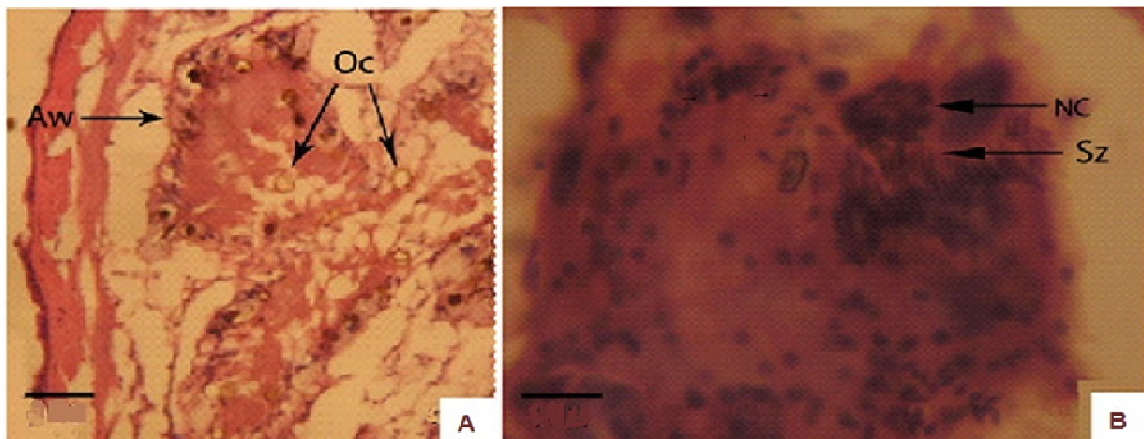


Plate 2. (A) Ovotestis of high egg state with many oocytes (Oc) within the acinar wall (Aw) compared to (B) with predominantly spermatogenic stages seen at other reproductive states. NC, nurse cell; Sz, spermatozoa. Scale bar = 10 μ m

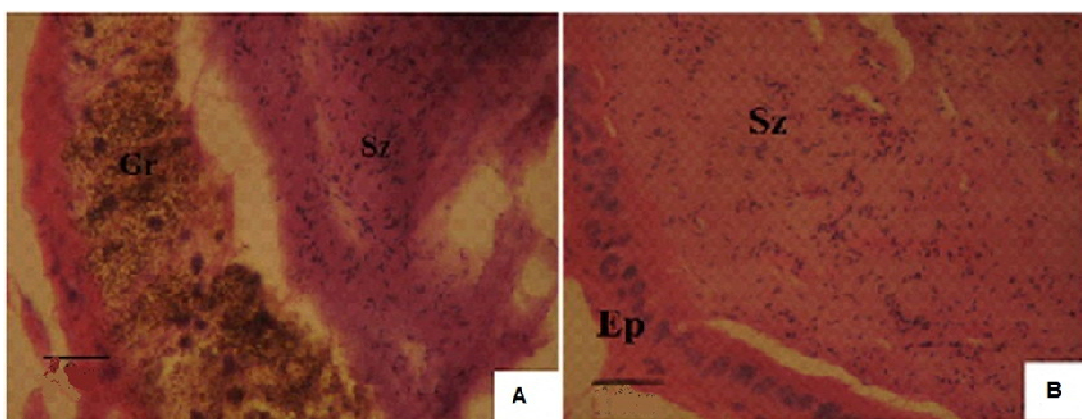


Plate 3. The lumen of the hermaphroditic duct contained spermatozoa (Sz) at all the reproductive states. The epithelial (Ep) cytoplasm also contained large quantity of granules (Gr) in the high egg state (A) compared to other reproductive states (B). Scale bar = 10 μ m

Albumen Gland: The albumen gland sections revealed the characteristics aggregation of secretory follicles. Group of 7-10 cells formed a circular follicle entrapping a luminal space in between. The follicle size was much smaller in the low mating state with relatively wider lumen compared to the post reproductive state with slightly larger follicle size and relatively smaller lumen. The follicle size was much bigger in the high mating, high egg and gravid states with larger cells filled with secretion.

Prostate gland: The prostate gland consisted of 3 -5 follicles each containing numerous acini. Each acinus contained about 10 radially arranged cells. The cells were tightly arranged in the low mating state whereas they form a central lumen that was filled with colloidal secretion in the high egg and gravid states. In the post reproductive state the cells appeared flaccid with an empty lumen leading to distorted acini (Plate 4).

Uterus: The uterine sections (Plate 5) showed a thin epithelium bounding the long lumen. Adjacent to the epithelium was the connective tissue layer that was twice as thick in the high egg, high mating and gravid states compared to the low mating and post reproductive states. The expanded connective tissue layer in the former appeared to straighten the epithelium to form a narrow lumen. In the later however the lumen was wider and the epithelium formed many invagination into the connective tissue layer

Spermatheca: The spermatheca of the low mating and post reproductive states showed a well folded epithelium compared to a straightened epithelium observed in the high mating, high egg and gravid states (Plate 6). In the high egg state the spermatheca lumen contained granular substances that appeared similar to the one observed in the epithelial wall of hermaphroditic duct of high egg state.

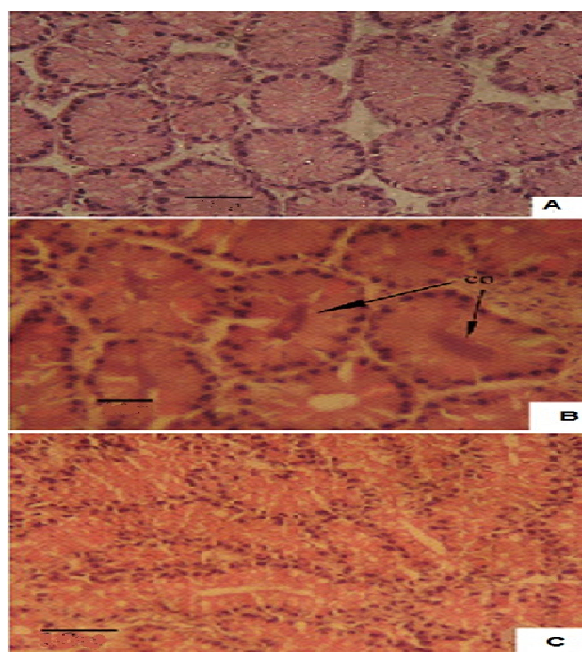


Plate 4. The acini of the prostate gland showing the lack of lumen in the low mating state (A), lumen filled with colloidal secretion (co) in the high egg and gravid states (B) and disrupted acini with empty lumen in the post reproductive state (C). Scale bar: A, B, C =10 μ m

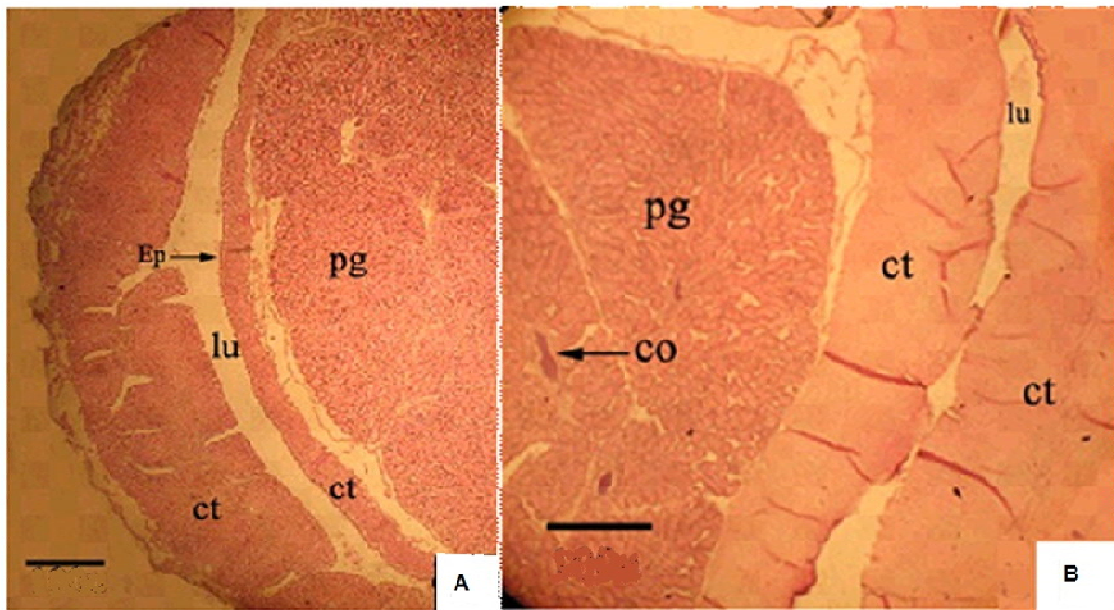


Plate 5. The connective tissue layer (ct) of the uterine wall was much reduced in the low mating and post reproductive states (A) than in the active reproductive states (B). Also in these active states colloids (co) could be seen in the follicular duct of prostate gland (pg) while the uterine lumen (lu) was also comparatively tighter. Scale bar = 100 μ m

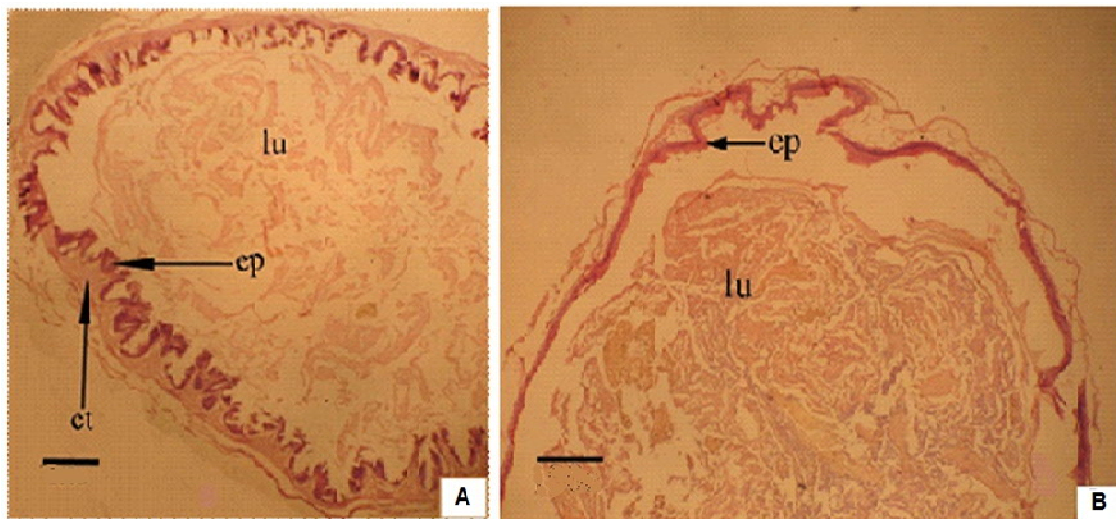


Plate 6. Cross section of the spermatheca at post reproductive state (A) and high mating state (B). Note the invagination of the epithelium (ep) into the connective tissue (ct) in (A) compared to the straightened epithelium in (B). lu, is the lumen of the spermatheca containing unidentified substance. Scale bar = 100 μ m.

Histochemical observations

Table 1 shows the summary of results of the histochemical staining of the different reproductive organs while Plate 7 and 8 are photomicrographs of representative samples of the histochemical reactions.

Table 1. Summary of histochemical reactions of reproductive organs of *A. marginata ovum* at different reproductive stages.

Reproductive organ	Test	Reproductive State				
		Low mating	High mating	High egg	Gravid	Post reproductive
Ovotestis	Glycogen	+	+++	+++	++	++
	Alkaline phosphatase	+	+++	++	++	++
	Protein	++	++	++	++	++
	Mucin	0	0	0	0	0
Hermaphroditic duct	Glycogen	+	++	+++	++	++
	Alkaline phosphatase	+	++	+++	++	++
	Protein	++	++	+++	++	+
	Mucin	+/-	+/-	+/-	+	+/-
Albumen gland	Glycogen	0	+++	+++	+++	+/-
	Alkaline phosphatase	+/-	++	++	++	0
	Protein	++	++	++	++	++
	Mucin	0	+/-	+/-	+	+
Prostate gland	Glycogen	++	+++	+++	++	++
	Alkaline phosphatase	++	++	+++	++	++
	Protein	++	++	++	++	++
	Mucin	++	+++	++	+++	++
Uterus	Glycogen	+	+++	+++	++	++
	Alkaline phosphatase	+	++	++	++	++
	Protein	++	+++	+++	++	++
	Mucin	+	+	++	++	+
Spermatheca	Glycogen	0	++	++	++	+
	Alkaline phosphatase	0	++	++	++	+/-
	Protein	++	++	++	++	++
	Mucin	0	++	++	++	+/-

+++ strong reaction; ++ moderate reaction; + weak reaction; +/- uncertain reaction; 0 negative reaction

Ovotestis: The acinar epithelium and interacinar connective tissue stained positively for glycogen, alkaline phosphatase and amino group but not mucin. At high mating state spermatocytes and spermatozoa were strongly stained for glycogen and alkaline phosphatase. The periphery of the oocytes was also similarly strongly stained for glycogen while the cytoplasm took weak staining for alkaline phosphatase in the high egg state.

Hermaphroditic duct: The epithelium and the spermatozoa filled lumen were positive, and very strongly so at high egg state, for glycogen, alkaline phosphatase and protein. In the high egg and gravid states the epithelium contained large quantity of granules that did not pick up glycogen stain. Reaction for mucin was not confirmed as the different slides showed conflicting weak positive and negative results.

Albumen gland: The albumen canal and the secretory follicles were filled with secretion that stained strongly for glycogen and protein at high mating, high egg and gravid states. The epithelium of the albumen canal and the intrafollicular connective tissue also stained moderately for alkaline phosphatase and protein. Mucin staining was partly visible in the gravid and post reproductive states and either negative or uncertain at

other states. Glycogen and alkaline phosphatase were unconfirmed in the albumen sections of low mating and post reproductive states.

Spermoviduct: The uterine epithelium and the acinar epithelium of the prostate gland were positively stained for both glycogen and alkaline phosphatase, and more strongly in the high mating and high egg states. The subepithelial connective tissue in the uterus and prostate gland were also positively stained for protein and mucin in all the reproductive states, with varying strength from weak to strong reactions.

Spermatheca: In the low mating state the spermatheca epithelium and lumen showed negative reaction for glycogen, alkaline phosphatase and mucin while the sub epithelial connective tissue stained moderately for protein. But in the high mating, high egg and gravid states, the epithelium was moderately stained for glycogen and alkaline phosphatase, while the subepithelial stained positive for protein and mucin. Alkaline phosphatase and mucin presence were doubtful in the post reproductive state. The spermatheca lumen in the high egg and gravid states was filled with granules similar to the one observed in the epithelium of hermaphroditic duct at the same reproductive state.

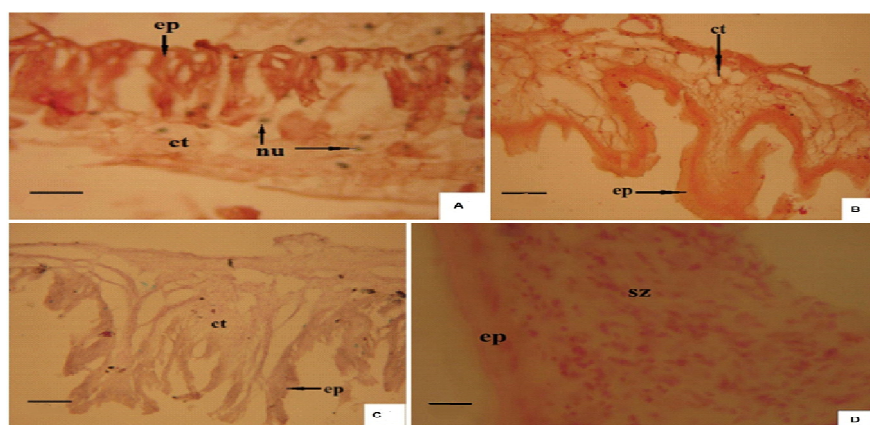


Plate 7. A) The uterine epithelium and the underlying multinucleated connective tissue were strongly positive for glycogen staining especially in the active reproductive states. B) Cross section of the spermatheca at high mating state showing strong epithelial staining for glycogen while the adjacent connective tissue was moderately positive. C) Section of the spermatheca at low mating stage showing negative reaction to glycogen staining. Note that both the epithelium (ep) and connective tissue of the well invaginated wall were negative. D) The epithelium of the hermaphroditic duct and the spermatozoa within its lumen were both strongly stained for glycogen at high mating state. (ep, epithelium; ct, connective tissue; nu, nucleus). Scale bar = 10 μ m.

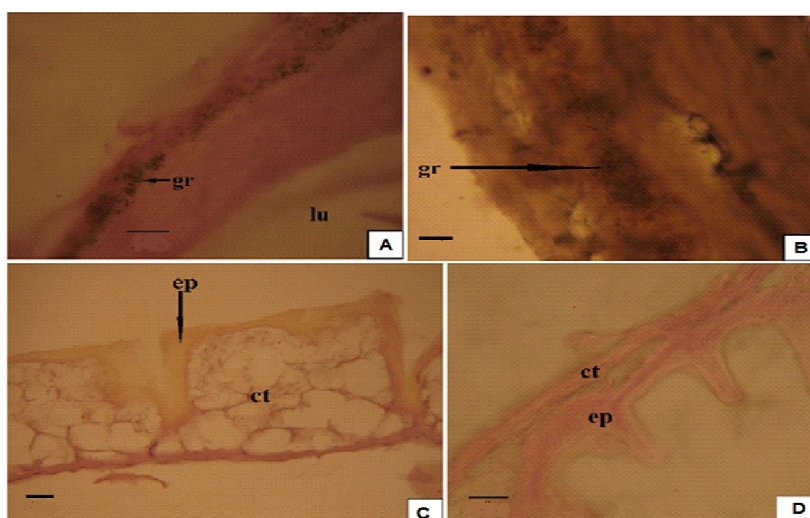


Plate 8. Cross sections of hermaphroditic duct at gravid state showing granular deposit (gr) in the epithelium which was neither positive for glycogen (A) nor alkaline phosphatase (B). The cross section of the spermatheca showed epithelium (ep) positive for mucin at high egg state (C) and for protein group at high mating state (D). Note that in (C) the connective tissue (ct) was negative for mucin staining. Scale bar = 20 μ m.

DISCUSSION

The reproductive cycle in *A. marginata ovum* which is reflected as morphological changes in the reproductive system is season dependent (Egonmwan, 2004; 2007a). This is also true for many other Stylommatophoran pulmonates (Albrecht *et al.*, 1999; Horn *et al.*, 2005). The results here have indicated that the morphological changes are also associated with physiological changes. The cycle appears to start when maturing juvenile snails begin to produce spermatozoa thus entering the mating readiness state and

later oocytes to be in egg production state. When simultaneous mating occur and a clutch of eggs is laid, it is not known whether the snail enter the post reproductive state immediately or remain in the egg production state until all the clutches are laid during the season. *A. marginata ovum* is reported to lay an average of 8-10 clutches of eggs per year (under non-seasonal environment) or 2 – 4 clutches per breeding season (where seasonal environment prevail) with an interval of about 30 days between clutches (Plummer, 1982).

The occurrence of spermatocytes and/or spermatozoa in the ovotestis of *A. marginata ovum* at all the reproductive states, including low mating (juvenile in most cases) is consistent with the protandrous nature of achatinidae (Ngowsiri *et al.*, 1989; Egonmwan, 2004), while the preponderance of oocytes in the ovotestis at high egg state during which mature spermatozoa were not evident could be a physiological guidance against self fertilization in the snail. Ngowsiri *et al.* (1989) also reported that oocytes production fluctuated with season in *Achatina fulica*. The spermatozoa aggregate in the lumen of hermaphroditic duct prior to ejaculation. In the high egg state were seen extensive granular deposit in the epithelial cytoplasm of the hermaphroditic duct. Similar granular deposit was observed in the lumen of spermatheca of high egg and gravid snails. It is opined that there may be a link between these separate granular deposits found at different locations but in the same reproductive state. Since achatinidae do not produce spermatophore it is not unlikely that the granular deposit in the hermaphroditic duct perform some functions (nutrition, capacitation or transportation) on the ova or spermatozoa and then find its way to the spermatheca (either its own or that of the copulating partner). In the spermatophore producing pulmonates such as *Helix pomata* (Lind, 1973), *Athoracophorus bitentaculatus* (Burton, 1978) and *Arianta arbustorum* (Haase and Baur, 1995) the spermatozoa is bound into a mass by the mucous secretion from the hermaphroditic duct and prostate gland and the mass ejected via the vas deferense into the spermatheca of copulating partner from where the spermatozoa are released for fertilization. The major function of the spermatheca is still not very clear in non spermatophore species like achatinidae.

The albumen gland enlarges considerably at high mating and high egg production states. This is due to its secretory activity during these stages. The albumen gland secretes perivitelline fluid which is added to the fertilized oocyte to nourish the developing embryo *in ovo* (Mead, 1950; Ghose, 1963). The secretory follicles of the albumen gland in *A. marginata ovum* at active (breeding) stages react strongly to Best carmine and Bromophenol indicating the presence of glycogen and protein, respectively. Since Best carmine reaction signifies both glycogen and galactogen we were unable to confirm if the albumen secretion in *A. marginata ovum* is glycogen or galactogen. However, many previous reports have confirmed galactogen as the major secretion of the albumen gland in other pulmonates: *Arion ater* (Smith, 1965); *Philomycus carolinianus* (Kugler, 1965); *Lymnaea Stagnalis* (Benita *et al.*, 1970); *Achatina fulica* (Ramasubramaniam, 1979).

The relatively thin spermoviduct in the low mating and post reproductive snails were engorged with secretions at high mating and high egg/gravid states leading to a significant increase in the size of the spermoviduct. The evidence of secretion at the active reproductive states in *A. marginata ovum* was seen in the sections across the prostate gland. Whereas the acini appeared turgid with no luminal space in the low mating state and flaccid with empty lumen in the post reproductive state, the acini lumen as well as interacini ducts in the high mating and high egg states contained colloidal secretions. The folliculi acini and uterine wall stained strongly for glycogen and alkaline phosphatase, especially in the high mating and high egg states, but also they were sites for significant mucin secretion. Ramasubramaniam (1979) had also reported the presence of acid mucopolysaccharide in the apical

uterus, and lipoprotein in the basal uterus and prostate gland of *Achatina fulica*.

The significance of a highly folded epithelial wall in the spermatheca of low mating and post reproductive snails could not be presently determined. However a plausible reason could be that the straightened epithelial wall of the high mating and high egg states was a direct result of distention caused by the fluid/gel materials that filled the spermathecal lumen at these states. Whatever is the function of the spermatheca or its content, histochemical results showed that there was little or no secretory function at non active compared to the active reproductive states in *A. marginata ovum*. Generally glycogen and alkaline phosphatase activities were associated together throughout the epithelium of the reproductive system of *A. marginata ovum* and they were more strongly indicated in tissues that are intimately connected to the growth and development of gametes. Alkaline phosphatase activity is a manifestation of energy breakdown and thus its association with glycogen presence is expected.

In conclusion we observed that morphological variation in the secreting glands of the reproductive system of *A. marginata ovum* is closely associated with changes in the functional secretory activities of the gland. The reproductive system of snails in the non active reproductive states (low mating and post reproductive) showed little or no secretory activity compared to the active reproductive states (high mating, high egg and gravid).

REFERENCES

Albrecht, E.A., Carreño, N.B. & Castro-Vazquez, A. 1999. A quantitative study of environmental factors influencing the sea-

sonal onset of reproductive behaviour in the South American apple-snail *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Journal of Molluscan Studies*, **65** (2): 241-250.

Benita, P., Marijke, D. & Boer, H.H.

1970. Histological and histochemical observations on the reproductive tract of the hermaphrodite pond snail *Lymnaea stagnalis* (L.). *Netherlands Journal of Zoology*, **21** (2):180-201.

Breckenridge, W.R. & Fallil, S.F.M.

1973. Histological observations on the reproductive system of *Achatina fulica* (Gastropoda, Pulmonata, Stylommatophora). *Ceylon Journal of Science, (Bio. Sci.)*, **10** (2): 85-120.

Burton D.W. 1978. Anatomy, histology and function of the reproductive system of the the Tracheopulmonate slug *Athoracophorus Bitentaculatus* (Quoy and Gaimard). *Zoology Publications from Victoria University of Wellington*—Nos. 68, 69 and 70.

Egonmwan, R.I. 2004. Maturation timing in the land snails *Archachatina marginata ovum* (Pfeiffer) and *Limicolaria flammea* (Muller). *Invertebrate Reproduction and Development*, **46**: 159–171.

Egonmwan, R.I. 2007a. Gross anatomy and characteristics of the reproductive system of the land snail *Archachatina marginata ovum* (Pfeiffer) during the breeding season. *Tropical Science*, **47**(2): 57–64.

Egonmwan, R.I. 2007b. Gross morphology and ultrastructural study of the albumen gland of the land snail *Archachatina marginata ovum* (Pfeiffer) (Pulmonata: Achatinidae). *Pakistan Journal of Biological Sciences*, **10**(2): 322-327

- Ghose, K.C. 1963.** Reproductive system of the snail *Achatina fulica*. *Proceedings of the Zoological Society of London*, **140**:681-695.
- Haase, M. & Baur, B. 1995.** Variation in spermathecal morphology and storage of spermatozoa in the simultaneously hermaphroditic land snail *Arianta arbustorum* (Gastropoda: Pulmonata: Stylommatophora). *Invertebrate Reproduction and Development*, **28** (1):33-41.
- Horn, A.C.M., Achaval, M, & Zancan, D.M. 2005.** The annual reproductive cycle of the snail *Megalobulimus abbreviatus* (Bequaert, 1948) (Gastropoda: Pulmonata). *Brazilian Journal of Biology*, **65**(3): 459-467.
- Kugler, O.E. 1965.** A morphological and histochemical study of the reproductive system of the slug, *Philomycus carolinianus* (Bosc). *Journal of Morphology*, **116**: 117–131.
- Lind, H. 1973.** The functional significance of the spermatophore and the fate of the spermatozoa in the genital tract of *Helix pomatia* (Gastropoda: Stylommatophora). *Journal of Zoology*, **169**: 39–64.
- Mead, A.R. 1950.** Comparative genital anatomy of some African achatinidae (Pulmonata). *Bulletin of the Museum of Comparative Zoology at Harvard College*, **105**: 219 – 291.
- Ngowsiri, U., Sertarugsa, P., Sobhon, P., Kruatrachue, M., Chavadej, J. & Upatham, S.1989.** Development of and seasonal changes in the reproductive system of *Achatina fulica*. *Journal of Science Society of Thailand*, **15**:237-249.
- Pearse, A.G.E. 1960.** *Histochemistry, Theoretical and Applied*, 2nd edition, Churchill, London, England.
- Plummer, J.M. 1982.** Reproductive potential and longevity in *Archachatina marginatovum*. *Journal of Molluscan Studies*, **48**: 366–367.
- KRamasubramaniam, 1979.** A histochemical study of the secretions of reproductive glands and of the egg envelopes of *Achatina fulica* (Pulmonata: Stylommatophora). *International Journal of Invertebrate Reproduction*, **1** (6): 333-346.
- Sheehan, D.C. & Hrapchak, B.B.** Theory and Practice of Histotechnology. 2nd ed. Y. B. Medical Publishers Ltd. Pp 481.
- Smith, B.J. 1965.** The secretions of the reproductive tract of the garden slug *Arion ater*. *Annals of the New York Academy of Sciences*, **118**: 997-1014.

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