DOCTOR OF PHILOSOPHY

The biology of reproduction in two British pulmonate slugs.

Nicholas, J.

Award date:
1984

Awarding institution:
Bangor University

THE BIOLOGY OF REPRODUCTION IN TWO BRITISH PULMONATE SLUGS

by

Jane Nicholas

A thesis submitted in partial fulfilment of the requirements for the degree of Philosophiae Doctor in the University of Wales.

In three volumes

VOLUME I

School of Animal Biology,
University College of North Wales,
Bangor, Gwynedd,
United Kingdom.

October 1984.

ACKNOWLEDGEMENTS

My sincere thanks to Dr. N. W. Runham for his advice and encouragement throughout this work. I should also like to thank Professor J. M. Dodd for providing the laboratory facilities and materials in the Department of Zoology.

I am grateful to Mr. D. A. Davies for his technical advice and assistance in the operation of the scanning and transmission electron microscopes and to Mrs. A. Buckland for her assistance in preparing some of the material for electron microscopy.

I am indebted to Mr. East (N.E.R.C. Unit of Larval Biology) for running the thin-layer chromatograms.

I acknowledge the help of Mr. J. Larsson in producing copies of the photographic material and I extend my thanks to Miss M. Mobed, Mrs. J. Rosario and Miss H. Spark for patiently typing this thesis.

Finally, I wish to thank my husband, Neil, and my parents for their constant support throughout this study.

CONTENTS

ABSTRACT

The biology of reproduction in two British pulmonate slugs.

The biology of reproduction has been studied in two British slugs, Deroceras reticulatum (Müller) and Arion hortensis (Férussac).

The morphogenesis, histology and ultrastructure of the carrefour and anterior genital ducts have been described and discussed in relation to their function.

The carrefour morphology of other slugs has been examined. The limacid carrefour is comparable to the basic stylommatophoran arrangement but in the Arionidae it is much simplified. It is concluded that the Arionidae could be an early offshoot from the main line of stylommatophoran evolution.

Evidence for the environmental control of reproduction in pulmonates has been reviewed. Experimental studies indicate that reproductive development and the onset of maturity are controlled by photoperiod. Short days retard and long days accelerate growth and reproductive development. Subsequent reproductive activity is unaffected by photoperiod.

Temperature, humidity and food availability have an immediate effect on egg-laying. In general, conditions favourable to the survival of the embryos enhance egg-production. None of these factors induce courtship and copulation but, generally, adverse conditions are inhibitory. Under apparently constant conditions the animals displayed periods of reproductive activity suggesting an endogenous rhythm. This may contribute to the synchronization of breeding cycles in the field.

The behavioural and functional changes during courtship, copulation and egg-laying have been described. During courtship the sperm's movement along the reproductive tract follows a consistent chronological pattern. At copulation the sperm packages are inserted directly into the entrance of the bursa copulatrix, but only sperm released before the package has completely entered the bursa travels up the reproductive tract. This sperm is not stored in the carrefour but passes through into the seminal vesicle where it mixes with the animal's own sperm.

At egg-laying the oocytes accumulate in the carrefour although fertilization does not necessarily occur there.

The incidence of self-fertilization in the pulmonates is discussed.

INTRODUCTION

There are three major slug families represented in Britain, the Arionidae, the Limacidae and the Testacellidae. Of these the carnivorous Testacellidae are rarely observed, usually remaining underground or beneath leaf litter where their prey is most abundant. Arionid and limacid slugs however, are frequently seen in gardens, fields and woodlands throughout the British Isles. They are herbivorous, feeding on a great variety of growing plants, decaying vegetable matter and fungi, and consequently they can become a serious pest on cultivated land. To reduce their effect on crops various control methods have been employed. It is possible to minimize slug damage by modifying the crop husbandry, by introducing natural predators and by applying lethal or disabling chemicals, but, for optimal results, it is essential to relate these forms of control to the animals' life cycles. For this reason research is carried out on all aspects of slug morphology and behaviour.

The majority of pulmonates are hermaphrodite. Three types of hermaphroditism occur, sequential, consecutive and simultaneous. In sequential and consecutive hermaphroditism a male phase usually precedes a separate female phase. This is known as protandry. Protogyny, where the animal first functions

as a female, is rare. In these two groups the gametes and the accessory sex organs are resorbed, or enter a resting state, before the alternative sexual phase commences.

In simultaneous hermaphrodites the gonads contain both male and female gametes, although in some species these may occupy separate locations within the gonad. The male and female tracts and associated accessory sex organs also develop simultaneously, but there is considerable variation in timing and in many simultaneous hermaphrodites the animals function as males before they are capable of female activity e.g. Arion ater (Smith, 1966). In others, e.g. Deroceras reticulatum , although there is a tendancy towards protandry, egg-laying can precede the production of sperm packages (Runham and Laryea, 1968). With functional sperm and ova present in the gonad at the same time, self-fertilization is possible. In simultaneous hermaphrodites there are however clear adaptations to reduce its occurrence.

This study looks at reproduction in two species of slug, Deroceras reticulatum (= Agriolimax reticulatus)

(Müller) and Arion hortensis (Férussac). D.reticulatum belongs to the family Limacidae which is characterized by a dorsal keel, a posteriorly-positioned pneumostome and a concentrically-ridged ('finger-print') mantle (Fig.1a). A small calcareous shell is enclosed within the mantle. Adult D.reticulatum may reach 5 cm in length, when fully extended, and are usually a pale greyish-brown colour with darker patches speckling the dorsal surface. The foot is cream, darkening towards the midline, and the mucus is normally clear, although when aggravated a white, sticky, alarm mucus is produced. In Britain breeding is continuous throughout the year with seasonal peaks in egg-laying.

In contrast, A.hortensis is a member of the family Arionidae which is recognized by the anteriorly-positioned pneumostome, wide foot fringe, distinct caudal mucus gland and truncated tail (Fig. 1b). The shell is reduced to discrete granules within the mantle. Adult A. hortensis are 2 to 3 cm long and dark grey in colour with a longitudinal black stripe running down either flank. The foot and mucus are yellow or orange. These slugs have an annual life cycle, breeding in summer and maturing within the year.

The reproductive tracts of D.reticulatum and A.hortensis have the same basic morphology (Fig. 2). The gonad has an acinar structure, each acinus opening into an efferent ductule which leads into the hermaphrodite duct. The hermaphrodite duct functions as a seminal vesicle and becomes white and swollen with the stored sperm. It passes into a complicated area known as the carrefour. The ducts from the albumen gland open here and it is thought that this area regulates the passage of sperm, fertilized ova, and albumen into the common duct. The common duct is a highly glandular region of the reproductive tract, composed of fused male and female ducts. The male duct is associated with the prostate gland, the female duct with the oviducal gland. Anteriorly the common duct bifurcates to form the vas deferens and free oviduct which pass into an evertile copulatory organ. The bursa copulatrix, a simple, digestive, sac-like diverticulum, opens near the entrance of the oviduct.

In D.reticulatum the copulatory organ is an expanded sac known as the penial mass. The vas deferens enters near its distal end and here the penial mass elaborates to form the trifid appendage. The base of the penial mass, the duct of the bursa copulatrix and the oviduct all open into the genital atrium.

In A.hortensis the vas deferens passes directly into a broader region known as the epiphallus. The epiphallus, bursa duct and free oviduct enter a short upper atrium which passes immediately into a highly glandular lower atrium.

In both species the atrium opens arteriorly, on the right side of the body, via the genital opening.

These two simultaneous hermaphrodites have a high degree of fusion of male and female ducts and the movement of gametes during copulation and egg-laying poses many interesting questions. What routes do the gametes take during these processes? What are the contributions of the accessory sex organs? How is gamete movement and the activity of the accessory sex organs related? Do these animals successfully avoid self-fertilization, and if so, how? What factors stimulate copulation and egg-laying?

To attempt to answer these questions it is first essential to establish the morphology and morphogenesis of the reproductive tract, paying particular attention to the carrefour and copulatory apparatus. Ultrastructural details of these areas provide necessary information concerning their function and this must be related to the animals' behaviour. Breeding colonies were set up in the laboratory and their responses to

different environmental conditions were recorded. In this way the effect of the environment on reproduction was assessed and the stimuli for gamete maturation, ovulation, egg-laying and copulation could be postulated.

MATERIALS AND METHODS

D.reticulatum and A.hortensis were either collected from the field in the vicinity of Bangor, or taken from stocks maintained in the department. Slugs reared from eggs laid in captivity, provided material for morphological study.

Animals were anaesthetized with solid carbon dioxide using a technique established by Bailey (1969).

Light microscopy

The size of the animal determined the level of dissection necessary for satisfactory fixation and penetration of the embedding medium. Newly-hatched specimens were prepared intact. For animals aged between two and four weeks, fixative was injected into the body cavity and the tail removed, while at five and six weeks the body wall was slit, exposing the internal organs. In older, larger animals the reproductive tract was dissected out. Since the albumen gland presents difficulties with sectioning, this was teased away, leaving the carrefour intact.

Tissues were fixed in Heidenhain's Susa, embedded in fibrowax and sectioned at 5µm. Weigert's iron haematoxylin, Biebrich scarlet-methyl blue variant

(Lillie, 1954), was used for routine staining. Additional staining with Alcian blue, pH 2.5 (Mowry), P.A.S. and bromophenol blue provided information on the secretory nature of the accessory sex organs.

Transmission electron microscopy

Tissues (< $1mm^3$) were fixed for 2 h at 0^oC in a mixture of 1 part 8% glutaraldehyde, 5 parts 2% OsO_4 and 4 parts veronal acetate buffer, adjusted to pH 7.5 (Wendelaar Bonga, 1970). Dehydration was through graded ethanols and the material was embedded in Araldite CY212. Semi-thin sections were stained with 1% toluidine blue in 1% borax, and ultra-thin sections with Reynold's lead citrate and uranyl acetate.

The grids were examined using a G.E.C. - A.E.I. Corinth 275 transmission electron microscope.

Scanning electron microscopy

Specimens were fixed for 3 h in 2% glutaraldehyde, buffered at pH 7.0 with 1.0 M sodium cacodylate, and

post-fixed for a further 1 h in 1% OsO_4 made up in the same buffer. Both fixatives were kept at $0^{o}C$ (after Nuwayhid et al., 1978). After dehydration in acetone the tissues were dried using the critical point method.

Each specimen was mounted, sputter-coated with gold and examined in an I.S.I. M-7 scanning electron microscope.

Thin layer chromatography

The atrial glands from 20 adult A.hortensis were homogenized in Hedon-Fleig saline and centrifuged at 10,000g for 20 mins. The clear layer floating on the surface of the cloudy supernatant was pipetted off. This is the aqueous extract (= solution A). The remaining supernatant and pellet were then homogenized with diethyl ether and re-centrifuged. The surface layer, containing small, yellow fat droplets, was removed (= solution B) and the ether extraction was repeated on the remaining supernatant and pellet. The final supernatant was decanted (= solution C).

Throughout the extraction the tissues were kept at 4°C and the final aqueous and ether extracts were stored at -20°C.

After thawing, the solvent was removed under vacuum with chloroform/ether, leaving a concentrated extract. The chromatographic plates (20 x 10cm) were coated with cellulose (300MN) to form a thin layer 250 to 300μm thick. The plates were loaded with 10μl of each concentrated sample, together with a standard containing equal proportions of cholesteryl oleate, methyl oleate, triolein, oleic acid and cholesterol. They were then placed in a continuous flow chromatography tank containing a solvent comprising petroleum ether : diethyl ether : glacial acetic acid, in the ratio 85 : 15 : 1. The tank was saturated with solvent vapour.

After one hour the plates were removed, dried at room temperature and viewed under ultra violet light. The spots were visualized by exposure to iodine vapour.

Stages of development

Runham and Laryea (1968) showed that there is continuous sequential development of the reproductive system. The weight of the animal is no indication of sexual maturity and cannot be used to assess an

animal's reproductive state. The development of the gonad, however, is closely related to the development of other parts of the reproductive tract. Therefore gonadal development is used to indicate the degree of maturation and, for convenience, has been subdivided into 8 stages labelled A to H, i.e. undifferentiated, spermatocyte, spermatid, early and late spermatozoon, early and late oocyte and post-reproductive.

A. Undifferentiated stage

The gonad is a solid mass of small, undifferentiated cells.

B. Spermatocyte stage

Acini have budded out from the central mass of cells and some or all of the following cell types may be present: Spermatogonia (small nuclei with one, or occasionally two, nucleoli), primary spermatocytes (large nuclei with a dense chromatin network), secondary spermatocytes (small nuclei with relatively large amounts of cytoplasm), oocytes (large cells with prominant nuclei containing very large nucleoli) and Sertoli cells (very large nuclei with fine granular chromatin). Oocytes and Sertoli cells are usually attached to the acinar wall.

The proportions of these cell types indicate whether development is at an early or late B stage.

C. Spermatid stage

Ducts are now present in the gonad and many secondary spermatocytes and spermatids (dense nuclei; sperm tails surrounded with cytoplasm) fill the centre of the acini.

D. Early spermatozoon stage

The acini now have a recognizable lumen which is filled with sperm tails. The sperm tend to lie in groups surrounding the Sertoli cells. A thick layer of spermatogonia and spermatocytes still lines each acinus.

E. Late spermatozoon stage

There is a clear lumen which may contain many unattached sperm. The layer of cells in the earlier stages of spermatogenesis is considerably reduced.

F. Early oocyte stage

Most of the oocytes are very large and covered by a thin layer of cells which forms the follicle. Sperm are present in large numbers.

G. Late oocyte stage

The amount of sperm is now very reduced.

H. Post-reproductive stage

A cuboidal epithelium covers at least part of the acinar wall. There is great variation in the amount of sperm and oocytes remaining in the acini.

This system of staging has been used throughout the present investigation, but it must be remembered that gonadal development is a continuous process and these stages are only a guide to the maturity of the animal. The later stages (F-H), in particular, show considerable overlap and once an animal is reproductively active the usefulness of staging is questionable.

Interpretation of cell activity

Any interpretation of cell activity is limited to the series of static images seen in the electron microscope, and further cytochemical investigations are necessary to positively identify the origin and destination of the numerous vesicles that are present in these cells.

The current literature concerning vesicle formation and membrane flow provides alternative routes which would account for the wide variety of vesicles, granules and lysosomes. These are summarized below and in Fig. 3:

The rough endoplasmic reticulum (rER) is continuous with the nuclear membrane (a). Proteins are synthesized on the ribosomes associated with the rER and pass into the lumen of the cisterna. At the periphery of the rER the ribosomes are lost and the sheet-like appearance of the flattened, orientated cisternae changes to a fine network of tubules (b) (diam. 0.03 - 0.06μm) (Novikoff *et al.*, 1971). This is the transitional ER (tER), also known as smooth ER, transitional sheet or GERL. It exhibits acid phosphatase activity (Novikoff *et al.*, 1971; Boutry and Novikoff, 1975; Novikoff, 1976; Novikoff *et al.*, 1977).

Vesicles bud off from the tER as transport or transitional vesicles. Coated vesicles, showing acid phosphatase acitivity, are primary lysosomes (c). They rapidly lose their clathrin coat and contribute to the formation of secondary lysosomes (d), multivesicular bodies (e) and phagocytotic vacuoles (f) (Alberts et al., 1983). Uncoated vesicles are directed towards the outer (cis) aspect of the Golgi stacks (g) (Novikoff et al., 1977), which selectively deposit osmium (Novikoff et al., 1971; Rothman, 1981). The inner (trans) element shows thiamine pyrophosphatase acitivity (Novikoff et al., 1971; Novikoff, 1976; Novikoff et al., 1977; Rothman, 1981).

The cis and trans portions of the Golgi apparatus are separate, but various observations suggest that proteins exported from the ER enter the stack at its cis face and exit from the trans surface. Transport through the stack is achieved by vesicles budding from cisternal rim to cisternal rim (h), by diffusion between closely apposed membranes, or, less likely, by each cisterna moving as an intact unit from cis to trans face (Rothman, 1981). It is now believed that the Golgi apparatus acts as a filter, refining and sorting the proteins (Rothman, 1981) and selective transfer by clathrin-coated vesicles (diam. 0.05 - 0.15μm) directs the different products and/or membranes to their correct destination (Rothman and Fine, 1980; Rothman, 1981;

Pearse and Brescher, 1981). In this way membranes with specific characteristics return to their parent organelle and many coated vesicles seen around the Golgi apparatus are probably returning to the ER (i).

There is no structural connection between the Golgi cisternae and the tER (Novikoff, 1977). Condensing vacuoles originate directly from dilated portions of tER (j) (Novikoff, 1971), directly from the trans element of the Golgi apparatus (k) (Novikoff, 1977) or by fusion of vesicles arising from the tER and the Golgi cisternae (l) (Nordmann et al., 1974). These vacuoles migrate towards the apex of the cell, fuse with the apical membrane and release their secretion by exocytosis (m). Redundant apical membrane is withdrawn by luminal invagination (n) or by endocytotic coated vesicles (o) (Geuze and Kramer, 1974). Luminal invaginations probably represent ghosts of extruded secretory granules. They may collapse and fragment, forming smaller vesicles (p), or they may give rise to multivesicular bodies by infolding and subsequent fission of their limiting membranes (q). The endocytotic coated vesicles become incorporated into secondary lysosomes (r) or multivesicular bodies (s), which suggests that lysosomal degredation of the cell membrane is necessary before it can be reutilized (Geuze and Kramer, 1974).

THE HERMAPHRODITE DUCT, CARREFOUR AND ALBUMEN GLAND

The hermaphrodite duct leads into a complex region of the reproductive tract known as the carrefour. This is not a discrete structure, but the area formed by the junctions of the hermaphrodite duct, the two albumen gland ducts and either the common duct in the Stylommatophora, or separate male and female ducts in basommatophoran snails. One or more diverticula may be associated with this region and their complexity varies throughout the Pulmonata.

The carrefour has aroused considerable interest since gametes must pass through this area during copulation and egg-laying. Shortly after copulation sperm is seen in at least one of the diverticula (Lind, 1973; Bayne, 1973) and they frequently contain orientated spermatozoa (Rigby, 1963, 1965; Mol, 1971), suggesting that this is the storage site for foreign sperm. In addition, the presence of fertilized ova in a separate diverticulum (Meisenheimer, 1907; Ikeda, 1937; Perrot, 1937) had led workers to believe that this was also the site of fertilization. The eggs normally contain a single zygote with a fairly constant volume of albumen or perivitelline fluid, and it seems likely that the carrefour coordinates the passage and packaging of the fertilized ova. Finally, the albumen secretion

appears very concentrated and it is thought that some dilution occurs in this region (Runham, 1984).

Thus, the carrefour plays a central role in controlling the movement of gametes at copulation and egg-laying. Despite this, relatively little is known about the pulmonate carrefour, principally because of the difficulties encountered in preparing and sectioning the associated albumen gland. Detailed observations on the morphology, morphogenesis and ultrastructure of the carrefour are necessary for a complete understanding of its involvement in reproduction. This study has concentrated on D.reticulatum and A.hortensis but a brief survey of the carrefour structure of other limacid and arionid slugs suggests that these findings are constant within the two families.

MORPHOLOGY AND MORPHOGENESIS OF THE CARREFOUR REGION IN D.RETICULATUM AND A.HORTENSIS

1. Morphology of the mature hermaphrodite duct

The morphologies of the hermaphrodite ducts of D.reticulatum and A.hortensis are similar (Fig. 4).

The duct extends from the gonad to the carrefour (Fig. 2) and for most of its length it functions as a seminal vesicle (Figs. 5 & 6).

Small, efferent ductules leave the gonad and converge to form a single, slender duct (Fig. 5) which immediately expands. It now appears cream-coloured due to the accumulation of stored sperm.

As the hermaphrodite duct approaches the carrefour it becomes empty and there is simultaneous reduction in diameter (Fig. 7). This slender region coils loosely before entering the carrefour.

A branch of the posterior genital artery runs alongside the hermaphrodite duct (Fig. 5) and terminates near the gonad. A small nerve lies close to this artery for most of its length.

2. The morphology of the mature carrefour in D.reticulatum

In mature animals the carrefour is enveloped by the large, pale albumen gland. When the two lobes of this gland are separated a small, ovoid diverticulum, 0.3 to 1mm. long, is seen lying in a shallow groove on the surface of the left hand lobe (Fig. 8). This diverticulum buds off from the carrefour at the junction with the hermaphrodite duct (Fig. 9).

The slender, anterior portion of the hermaphrodite duct describes a loose coil before entering a flattened, blind-ending pouch, which resembles a horseshoe in transverse section (Fig. 10). The duct passes into the pouch near its base, at the mid-point of the inner wall (Fig. 11). At the same level, a finger-like, blind-ending process buds off from the right-hand arm of the 'horseshoe', extending upwards and becoming partially surrounded by the pouch (Figs. 10 & 11). The outer wall of the pouch is highly folded.

At its base the pouch becomes irregularly lobed losing its characteristic horseshoe shape (Fig. 12). A 'carrefour' gland is present in this region, extending from the base of the pouch to the start of the common duct (Figs. 13 - 15), where it is associated with the male groove. The right and left albumen gland ducts open into the base of the carrefour at this junction (Fig. 14).

Upon entering the carrefour region the posterior genital artery branches into many fine blood vessels which vascularize the albumen gland (Fig. 16). The blood supply to the carrefour diverticula is very poor however, and only a few, small vessels are present around the pouch or finger-like process.

No major nerves supply this area and innervation of the carrefour is achieved by an extensive nerve plexus.

3. The morphology of the mature carrefour in A.hortensis

In contrast to D.reticulatum the carrefour of A.hortensis has a very simple structure (Fig. 17). The slender, anterior portion of the hermaphrodite duct coils loosely once or twice before straightening to form the descending limb of a simple loop (Figs. 18 & 19). As it nears the bend of this loop the duct widens and then turns back on itself to become the ascending limb (Figs. 19 & 20). There is little folding of the duct walls but as it passes out of the loop area it becomes irregularly lobed and receives two albumen gland ducts (Fig. 21) before passing into the common duct. A 'carrefour' gland extends from the proximal end of the ascending limb (Fig. 22), to the start of the common duct, where it is associated with the male groove.

This carrefour loop is surrounded by a connective tissue sheath which gives it the appearance of a single diverticulum, 0.5 to 1.5mm in length. It is embedded into the surface of the left lobe of the albumen gland (Fig. 8b).

As in D.reticulatum the carrefour is poorly vascularized (Fig. 23). Innervation is achieved by an extensive nerve plexus.

4. The morphology of the mature albumen gland

The albumen gland consists of two large lobes which open into the base of the carrefour via two separate, short ducts (Fig. 2). The left duct is slightly anterior to the right and the left-hand lobe is normally larger, with occasional small lobules.

In D.reticulatum the lobes are rounded and grey or cream in colour, becoming paler as the gland swells with accumulated secretion. The lobes of A.hortensis, however, are more elongated, tapering distally, and in some animals they extend back as far as the gonad. The gland has a looser structure and appears darker than that of D.reticulatum.

The albumen gland is well vascularized by branches of the posterior genital artery (Fig. 16 and 23) but no major nerves are present in this area.

5. Morphogenesis of the carrefour region in D.reticulatum

In newly hatched animals the reproductive tract is a simple undifferentiated tube extending from the gonad (stage A) to the genital opening (Fig. 24). By the late A stage a few cells have begun to proliferate approximately half-way along the reproductive tract (Fig. 25) . This proliferation results in an increase in the duct diameter and indicates the start of carrefour and albumen gland differentiation. It separates the region of the tract which will develop into the hermaphrodite duct from that developing into the common duct.

During the early B stage of development the undifferentiated hermaphrodite duct coils loosely once or twice just before it opens into the carrefour (Fig. 26a). Two small processes bud off at this junction (Fig. 27) and these correspond to the large horse-shoe shaped pouch and smaller diverticulum of the mature animal. The carrefour immediately expands into the rudimentary albumen gland (Fig. 28). At this stage there are no discrete albumen gland ducts and the left and right lobes are just simple swellings of the epithelium (Fig. 29). As the reproductive tract passes into the common duct it takes on the distinctive comma-shaped profile

which indicates the start of oviducal and prostate gland differentiation (Fig. 30).

Morphogenesis is complete by the end of the B stage. The hermaphrodite duct begins to increase in diameter by cell proliferation (Fig. 31) and the two small processes differentiate to form a complex ovoid structure, approximately 0.1 mm in length (Fig. 26b). One process expands to form a flattened pouch which is crescent-shaped in transverse section (Fig. 32). The hemaphrodite duct enters the carrefour near the base of this pouch at the midpoint of the inner wall (Fig. 33). At the same level the second process buds off from the right hand arm of the crescent as a short, thick diverticulum (Figs. 32 & 33). The lobes of the albumen gland expand (Fig. 34) and at the same time their junctions with the carrefour become constricted forming two narrow ducts. These ducts open into the base of the carrefour just before the start of the common duct.

Maturation of the carrefour proceeds throughout the C and D stages of development. The hermaphrodite duct begins to function as a seminal vesicle and the narrow portion adjacent to the carrefour become highly convoluted. The carrefour diverticula expand further and may become very lobed. There is proliferation of the albumen-, carrefour-, oviducal- and prostate gland secretory cells.

By the end of stage D the animals are fully mature and reproductive function is possible.

6. Morphogenesis of the carrefour region in A.hortensis

Morphogenesis of the reproductive tract of A.hortensis follows a similar time course to D.reticulatum.

In newly-hatched animals the reproductive tract is a simple undifferentiated tube (Fig. 35). Differentiation of the carrefour begins before the end of the A stage with proliferation of a few cells approximately half-way along its length (Fig. 36). This slight swelling separates the hermaphrodite duct from the common duct.

During the early B stage the hermaphrodite duct remains undifferentiated but in the carrefour region two diverticula bud off to form the rudimentary ducts of the albumen gland (Fig. 37a). These expand into two flattened sacs or lobes, a short left-hand one and a long, slender right-hand lobe. (Figs. 38 - 41). Shortly after leaving this junction with the albumen gland ducts the tract widens and passes into the common duct (Figs. 41).

During the late B stage the hermaphrodite duct elongates and, as it approaches the carrefour, loops back on itself to form the descending limb of the carrefour (Figs. 37b & 42). It then turns sharply into the ascending limb which widens as it approaches the junction with the albumen gland ducts (Fig. 43). The lobes of the albumen gland expand and elongate, particularly the right hand lobe which extends half-way along the hermaphrodite duct (Fig. 44). The base of the carrefour is irregularly shaped and passes quickly into the common duct which now assumes its characteristic comma shape (Fig. 45). The carrefour is surrounded by a thick, connective-tissue sheath.

Morphogenesis is now complete. Maturation of the carrefour proceeds throughout the C and D stages of development. By the end of the D stage the hermaphrodite duct begins to function as a seminal vesicle and the slender region adjacent to the carrefour becomes highly convoluted. The carrefour loop elongates and there is proliferation of the albumen-, carrefour-, oviducal- and prostate gland secretory cells.

7. Comparative morphologies of arionid and limacid carrefours

The carrefours of other limacid and arionid slugs were examined. The diverticula observed in D.reticulatum were also seen in D.caruanae, Limax marginatus, L.maximus, L.grossui, L. pseudoflavus, Milax Sowerbyi and M. budapestensis. The simple looped carrefour of A.hortensis was also found in A.ater ater, A. ater rufus, A.intermedius, and A.fasciatus. It would appear, therefore, that the morphology of the carrefour is consistant within these slug families.

ULTRASTRUCTURE OF THE REPRODUCTIVE TRACT IN NEWLY-HATCHED ANIMALS

In the hatchling, the reproductive tract is completely undifferentiated and lined by cuboidal epithelial cells with prominant central nuclei (Figs. 46 to 48). Each nucleus contains a single spherical nucleolus which is surrounded by irregular clumps of chromatin. Small tubular mitochondria are scattered throughout the cell and occasional, thin strands of rough endoplasmic reticulum (rER) are seen around the nucleus. The cytoplasm contains numerous polysomes, small clear vesicles (diam. 0.05 - 0.15μm) and occasional multivesicular bodies (MVBs). Desmosomal junctions are present at the apices of the cells. The

whole tract is surrounded by a thin connective tissue sheath, which consists of a simple collagen matrix containing scattered fibroblasts.

ULTRASTRUCTURE OF THE CARREFOUR REGION IN D.RETICULATUM

1. The mature hermaphrodite duct

The slender portions of the hermaphrodite duct are completely ciliated (Figs. 5 & 7), but as the duct widens into the seminal vesicle these ciliated cells become restricted to a single continuous band, one third to one half of the duct's total circumference, and unciliated cells differentiate around the remaining portion of the duct (Figs. 7 & 49).

The seminal vesicle

i) The ciliated cells

The ciliated epithelial cells are columnar and have rounded central nuclei containing small clumps of chromatin (Fig. 50). The nuclei are surrounded by large accumulations of glycogen which tend to displace the cell organelles apically.

Small concave Golgi bodies, consisting of 6-7 cisternae, lie immediately above the glycogen (Fig. 51), and coated vesicles with clear contents (av. diam. 0.05 μm) appear to bud from the tips of the slender, inner cisternae. The outer cisternae are distended and more irregularly shaped. Large, clear vacuoles are seen near the outer surface of the Golgi stack. A variety of vesicles are present throughout the cytoplasm; small, clear vesicles (diam. 0.05 - 0.1μm) accumulating near the apical membrane (Fig. 52) while larger electron-dense vesicles (max. diam. 0.4μm) and multivesicular bodies are seen in the sub-apical region (Figs. 52 & 53).

Cylindrical mitochrondria are concentrated at the apices, forming the mitochondrial cloud characteristic of ciliated cells (Figs. 50 & 52). Polysomes are scattered throughout the cytoplasm and longitudinally orientated bundles of microtubules and microfibrils are present around the periphery of the cell (Fig. 53).

The apical membrane bears both microvilli and cilia, the long, striated ciliary rootlets extending into the cytoplasm for 2μm (Fig. 52). A single lateral foot is clearly seen projecting from each basal body. Desmosomal junctions are present at the luminal surface forming a continuous band (0.3 - 0.4μm thick) around the apex of each cell. Zonulae adhaerentes extend for a

further 0.5 - 1μm. Hemi-desmosomes are frequently seen along the basal membranes.

ii) The unciliated cells

The shape of the unciliated cells alters as the duct fills with sperm. Newly differentiated cells are cuboidal (Fig. 54) (cell height 10 - 20μm) but they have a great capacity for stretching and as the pressure increases within the duct these cells become squamous (cell height 4 - 7μm) allowing for a six-fold increase in duct diameter (Fig. 55).

The prominent, central nuclei are very rich in chromatin and have a characteristic speckled appearance. Small Golgi bodies consisting of approximately five cisternae lie near the nucleus (Fig. 56). The cisternae are often very distended and are surrounded by clear-staining vesicles (diam. 0.05 - 0.2μm). Many of the smaller vesicles appear to have a fuzzy (clathrin) coat, and similar vesicles form short chains which are associated with the apical membrane. Large multivesicular bodies (diam. 0.3 - 0.5μm) accumulate in the sub-apical region (Fig. 57).

The apical membrane bears numerous microvilli (Fig. 57), but as the cells become squamous these are lost and the apices appear smooth, sometimes assuming a

scalloped appearance as stored sperm become partially embedded in the surface of the cell (Fig. 55). Completely phagocytosed sperm, at various stages of breakdown, are frequently seen in the cytoplasm (Figs. 54 & 58). The newly formed phagocytotic vesicles usually contain one or more multivesicular bodies.

Small mitochondra and isolated strands of rough ER are seen in the nuclear-Golgi region. There are no stores of glycogen in these unciliated cells.

Belt desmosomes (0.1 - 0.2μm thick) are present around the apex of each cell and a zonula adhaerens extends for a further 1.5μm. Hemidesmosomes are seen along the basal membranes.

In older animals large clear vacuoles develop between the epithelial cells (Fig. 59). The cytoplasm of these cells generally has a high concentration of clear vesicles of varying sizes.

iii) The connective tissue sheath

A thin layer of moderately-dense material, 40 - 70nm thick, closely follows the contours of the cells (Fig. 60). This is the basement membrane. Immediately underneath this, a layer of randomly-orientated collagen fibres (0.2 - 0.7μm thick) is surrounded by a band of

circular muscle and finally, an outer layer of longitudinal muscle (Figs. 50, 54 & 60). Elongated pigment cells are occasionally found between these two muscle layers and also at the periphery of the connective tissue sheath. A single, large nerve runs alongside the duct (Fig. 61) and isolated axons, filled with dense-cored neurotransmitter granules, may be seen.

The extracellular connective tissue matrix is composed of loose bundles of collagen within an amorphous ground substance.

The slender coiled region of the hermaphrodite duct

The ciliated cells of this region are similar to those seen in the seminal vesicle (Figs. 62 & 63), but as the duct nears the junction with the carrefour diverticula two changes occur. Firstly, the stores of glycogen disappear (Fig. 64). Then, as the duct opens into the carrefour the cells lose their cilia (Fig. 65) until the apical membrane bears only microvilli.

The connective tissue sheath surrounding this slender portion of the hermaphrodite duct is different to that of the seminal vesicle (Fig. 66). The layers of muscle become reduced and patchy and there is a considerable increase in the amount of nervous material. Peripheral neurons become more frequent as the duct

approaches the carrefour and their axons spread throughout the connective tissue forming an extensive nerve net (Fig. 67). The nerve cells establish an intimate relationship with the epithelium and axons, filled with dense-cored, neurotransmitter granules, are regularly seen in small pockets or grooves running along the basal plasma membrane (Fig. 68). This sheath is continuous with that enveloping the carrefour.

2. The carrefour diverticula

i) Of immature animals

The carrefour diverticula of immature D.reticulatum (late B/early C) are lined by small columnar, epithelial cells with prominant basal nuclei (Fig. 69). The nucleus occupies approximately 2/3 of the cell and contains a single nucleolus which is surrounded by irregular clumps of chromatin. Apically there are a few small mitochondria and clear vesicles but no identifiable Golgi apparatus is observed at this stage. The apical membrane bears stubby microvilli and the occasional basal body of a developing cilium is seen. There are desmosomal junctions at the apices of the cells while hemidesmosomes are present at regular intervals along the basal membranes. There is little interdigitation of the cells and the basal membranes

lack the complex infolding which is seen in the mature animal.

ii) Of mature animals

The epithelia of the two diverticula differ slightly from one another. The large flattened pouch is lined by tall, narrow, columnar epithelial cells and the prominant basal nuclei assume an elongated oval shape, filling the lower third of each cell (Fig. 70). The nucleus contains a single nucleolus which is surrounded by clumps of heterochromatin.

In the mature animal the cells are very active. The rER is filled with a pale-staining product and in some animals the cisternae appear quite swollen (Fig. 71). An extensive Golgi complex lies immediately above the nucleus and the stacks of 5 to 7 slightly-concave cisternae are surrounded by clear vesicles (diam. 0.05 - 0.15μm) (Fig. 72). Occasionally, small amounts of an electron-dense material are seen within the inner cisternae and a few dense-cored vesicles(diam. 0.05 - 0.1 μm) may be found in the neighbouring cytoplasm (Fig. 71). The outer cisternae are frequently distended and appear to be associated with larger clear vacuoles (max. diam, 0.25μm).

Small, electron-dense, secondary lysosomes are frequently seen near the Golgi apparatus (Fig. 72). Their size and distribution increases with age and in post-reproductive animals (stages G-H) approximately one in ten cells contain large, irregularly shaped lysosomal bodies.

A variety of vesicles are seen at the cell apex (Fig. 73). Electron-opaque granules (max. diam. 0.2μm) lie immediately beneath the 'mitochondrial cloud', some containing small dense inclusions which suggest that these may also be lysosomal. Numerous clear vesicles (diam. 0.05 - 0.25μm) accumulate near the cell surface, the smaller vesicles (diam. 0.05 - 0.1μm) frequently forming chains leading to or from omega bodies in the apical membrane (Fig. 74). The larger vesicles generally contain one or more of the smaller variety and appear to be young multivesicular bodies. The number of vesicular inclusions increases as the MVBs near the Golgi apparatus.

Tubular mitochondria with well-developed, laminar cristae are concentrated near the rER/Golgi zone and at the cell apex (Figs. 71 & 73). Longitudinally-orientated microtubules are present at intervals around the periphery of each cell (Fig. 72), and scattered polysomes give the cytoplasm a fine granular appearance.

The apical membrane is densely ciliated and bears numerous microvilli (Fig. 73). The cilia have a typical 9 + 2 configuration of the axoneme with long (2μm), striated rootlets. Along the lateral edges of the pouch lobes there is a band of unciliated cells, four or five cells wide, whose apical membranes bear only microvilli (Fig. 75). Unciliated cells are also observed at the junction with the hermaphrodite duct (Fig. 76), but they tend to lose their microvillar border and surface detail is reduced to irregular blips in the apical membrane. The unciliated cells contain fewer mitochondria at the apex. The number of vesicle-chains, associated with the apical membrane increases and MVBs are common (Figs. 75 & 76).

There is considerable folding of the longitudinal membranes and the cells are tesselated when seen in transverse section (Fig. 71). Belt desmosomes (0.3μm thick) are present around the apex of each cell and beyond this, small zonulae adhaerentes occur at regular intervals.

The degree of folding in the basal membranes increases with age, becoming very complex in post-reproductive animals (Fig. 77). Hemidesmosomes are present at frequent intervals.

The epithelial cells of the finger-like diverticulum tend to remain cuboidal and their round basal nuclei contain little chromatin (Fig. 78). The Golgi bodies are fewer in number but appear to be as active as those of the pouched diverticulum, and at the cell apex there are numerous small vesicles and several large MVBs. The rER, on the other hand, is more extensive and the cisternae ramify the perinuclear cytoplasm. In all other respects these cells are similar to those described above.

3. The connective tissue surrounding the carrefour diverticula

i) Of immature animals

The carrefour is ensheathed by a thick layer of connective tissue. Fibroblasts and indeterminate connective tissue cells are scattered throughout the loose collagen matrix, and at this stage large numbers of peripheral neurons and muscle cells are present (Figs. 79 & 80).

The developing neurons are large cells with several short, tapering axonal processes radiating out from the cell body (Fig. 81). There is a large prominent nucleus containing irregular clumps of chromatin (Fig. 82), while small, oval mitochondria, polysomes isolated rER

cisternae and numerous clear vesicles (av. diam. 0.06μm) are dispersed throughout the cytoplasm. The vesicles are produced by a tightly- stacked Golgi complex and MVBs may be present nearby. Large numbers of longitudinally-orientated neuro-tubules (diam. 0.02μm) are seen in the axons and clear vesicles (diam. 0.05 - 0.07μm) fill the swollen tips of some processes. The axons are slender and have a circular cross-section (diam. 0.16 - 0.32 μm). Bundles of up to 60 axons may be bound together by connective tissue to form the nerves of this developing plexus. At this stage these nerves appear small and insignificant (Fig. 83).

Small muscle fibres are present throughout the connective tissue. Those underlying the epithelium tend to follow the contours of the basal membranes, but elsewhere they show no obvious orientation or organization (Figs. 79 & 81).

ii) Of mature animals

With the increase in size and complexity of the carrefour diverticula there is a corresponding increase in the size and complexity of the connective tissue sheath (Figs. 84 & 85).

The nerve plexus has developed to such an extent that the large neurons and interconnecting nerves are now the major component of the sheath (Fig. 86). This has been achieved by the neurons increasing in size rather than number. Serial sections through the nerve plexus have shown that these are multipolar neurons, the rounded cell bodies tapering into 5 or 6 axonal processes. There is an irregularly-shaped, central nucleus containing small clumps of chromatin (Fig. 87). The cytoplasm is packed with clear, spherical vesicles, with an average diameter of 0.05μm (Fig. 88). These vesicles are produced by the compact Golgi bodies which lie near the nucleus, each consisting of a stack of 3 to 5 slender cisternae. Many cisternae contain an electron-dense secretion which is released into the cytoplasm as small, dense-cored vesicles (diam. 0.08 - 0.1μm) (Fig. 88). Only a small number of these dense-cored vesicles are seen. Cylindrical mitochondria and rough ER cisternae are found mainly at the periphery of the cell body, while numerous polysomes are scattered throughout the cytoplasm. In many neurons secondary lysosomes are present, their size and abundance increasing with age (Fig. 89).

The axonal processes vary in size. Some still appear small and insignificant (diam. 0.15μm), while others have diameters of nearly 2μm (Fig. 90). They are arranged in bundles, held together by connective tissue,

to form the prominant nerve trunks. Some nerves are made up of only 20 axons while the largest contain over 100. The increase in number and size of the axons has resulted in tight packaging and the largest axons have lost their circular profile, becoming polygonal (Fig. 90). The vesicles observed in the cell body extend into the axon cytoplasm. Their distribution varies and while some axons are packed with both clear and cored vesicles, others appear empty (Fig. 91). A few axon profiles contain clusters of dense granules, 0.02 - 0.03 μm in diameter (Fig. 91). These could not be identified. Other larger granular inclusions (diam. 0.15μm) are probably pigment granules, in the attenuated processes of pigment cells.

The tips of the axons are swollen and appear to form synapses with adjacent neurons (Fig. 92), and muscle cells (Fig. 93). The expanded ends are packed with the small clear vesicles, and the neuromuscular junctions, typically, contain many dense-cored vesicles as well.

Small, cylindrical mitochondria are occasionally seen in the larger axons, tending to aggregate at the swollen tips. Longitudinally orientated neurotubules (approx. diam. 10nm) extend throughout the axonal processes (Fig. 91) but are rare or absent in the synapse bulb.

At the base of the pouched diverticulum some neurons lie in pockets or infoldings of the basal membrane, coming into intimate contact with the epithelial cells (Fig. 94).

Muscle fibres are present throughout the connective tissue sheath. There is a tendency for those underlying the epithelium to follow the contours of the basal membrane but elsewhere they exhibit random orientation (Fig. 84). The muscle cells are very long and spindle-shaped. There is a central, slender nucleus which contains an inconspicuous nucleolus and aggregates of chromatin line the nuclear envelope. Short, cylindrical mitochondria are seen throughout the cytoplasm and at the periphery of each cell there is a network of smooth tubules which corresponds to the sarcoplasmic reticulum of vertebrate skeletal muscle cells (Fig. 95). Longitudinally orientated myofilaments run throughout the cell. There are two kinds, thick filaments with diameters of 0.03μm and thinner filaments approximately 0.01μm in diameter. Hemidesmosomes are seen at intervals along the cell membrane (Fig. 96).

Occasionally desmosomal junctions are observed between muscle cells and the basal membranes of the epithelial cells.

Pore cells are infrequently found in the connective tissue sheath of older animals (stages F-H). These are large irregularly-shaped cells which are characterized by having typical surface slits covering chambers in the surface cytoplasm (Figs. 16, 97 & 98) (Sminia, 1972). These chambers appear to be interconnected . The nucleus, with its prominant central nucleolus, is surrounded by an extensive Golgi complex. The Golgi stacks are made up of 4 to 6 cisternae which bud off clear vesicles (diam. 0.05 - 0.1μm) from their tips. These vesicles fuse, forming large pale-staining granules of up to 2μm in diameter. In addition to these secretory granules there are many secondary lysosomes which contain irregular electron-dense patches. In older animals the lysosomes are larger and may eventually fill most of the cell. A pale flocculent material, probably glycogen, lies between the granules. Due to the accumulation of lysosomes and secretory granules the rough ER tends to be restricted to the cytoplasm surrounding the nucleus and at the periphery of the cell.

Pigment cells are present at the periphery of the connective tissue sheath (Fig. 85) and occasionally lie alongside the large nerves at the heart of the nerve-net. They are slender, elongated cells which branch into several tapering processes and contain numerous electron-dense pigment granules (max. diam. 0.5

μm). The nucleus occupies most of the cell body and is surrounded by swollen rough ER and an inconspicuous Golgi apparatus. Bundles of longitudinally orientated microfilaments, glycogen granules and polysomes are seen throughout the cytoplasm.

Mucocytes are also present in the connective tissue sheath (Fig. 70). Their long necks push between the epithelial cells of the pouched diverticulum and mucus is released into the lumen. They never discharge into the smaller diverticulum. The fine structure of these cells was not recorded.

4. The carrefour gland

Differentiation of the carrefour gland begins during the early C stage of development. Mesenchyme cells in the thick connective tissue sheath surrounding the base of the carrefour, proliferate, and differentiate into secretory cells. There are no discrete collecting ducts and as the cells mature they force their way between the ciliated epithelial cells and release their secretion directly into the duct lumen (Fig. 99).

The irregularly-shaped basal nuclei stain densely due to the large amounts of chromatin dispersed throughout the nucleoplasm. There is a single prominent

nucleolus. The cytoplasm is packed with numerous Golgi bodies and swollen rER cisternae (Fig. 100) and both organelles are involved in the synthesis of large pale-staining secretory vesicles. The cisternae of the rough ER contain rod-like granules. These have a circular profile (diam. 0.02μm) with clear contents, and lie perpendicular to the membrane (Fig. 101). Each granule appears to be associated with a separate ribosome on the membrane surface but loses its distinctive shape as soon as it is released into the cisternal lumen. The rER cisternae are continuous with large, smooth-surfaced secretory vesicles (Fig. 102).

The Golgi bodies are usually cup-shaped (Fig. 103) and the cisternae are swollen and distorted by the accumulation of pale-staining secretion (Fig. 102). The cisternae bud off large vesicles which appear to fuse with each other and with the vesicles arising from the ER. Smooth cisternae with slender, tubular elaborations (diam. 0.04 - 0.06μm) of the membrane are frequently seen between the Golgi stacks and the secretory vesicles of the ER (Fig. 104). The tubules form close associations with the outer Golgi cisternae. This is probably transitional ER.

The secretory granules have fine, granular contents and accumulate at the apex of the cell.

Eventually the membrane ruptures and the secretion is released into the carrefour.

Small, cylindrical mitochondria are only seen clearly in immature cells. As the rough ER and Golgi apparatus develop the mitochondria are compressed between these organelles and become difficult to identify.

5. The developing albumen gland

By the end of the B stage of development the albumen gland consists of two simple sacs opening into the carrefour via short ducts (Figs. 34 & 105a). It is lined by unciliated epithelial cells with large prominant nuclei containing irregular clumps of chromatin (Fig. 106). Small, rounded mitochondria are scattered throughout the cytoplasm and the apical membrane bears occasional, stubby microvilli. Desmosomal junctions are present at the apices of the cells. At this stage the cells are completely unspecialized.

Maturation of the albumen gland begins during the early C stage. There are three phases of development, proliferation (Fig. 107), accumulation (Fig. 108) and secretion (Fig. 109).

i) The proliferation phase

Cell division and growth within the epithelium of the immature albumen gland results in the formation of numerous blind-ending tubules or ductules (Fig. 105b). Clusters of cells, probably mesenchymal in origin, then accumulate beneath the epithelium (Figs. 105c & 110).

The cells lining the original sac-like portion of the gland show no signs of differentiation or cell-division (Fig. 110). This region becomes the main collecting duct of the mature albumen gland.

The epithelial cells lining the ductules multiply rapidly and consequently they appear rather irregular in shape. Their prominant basal nuclei (Figs. 110 & 111) each contain a large, central nucleolus which is surrounded by clumps of chromatin. A few cylindrical mitochondria are scattered throughout the cytoplasm and isolated rough ER cisternae are seen around the nucleus. At the apical surface there are microvilli and an occasional developing cilium. Belt desmosomes (0.3µm thick) surround the apex of each cell and zonulae adhaerentes extend for a further 1µm.

As the sub-epithelial cells proliferate solid buds develop around the ductule (Fig. 112). These are the immature secretory cells. During this phase of

rapid cell division they are small and irregularly-shaped, with large, central nuclei. Each nucleus contains a prominant nucleolus which is surrounded by masses of chromatin, giving it the characteristic speckled appearance common to all glands associated with the reproductive tract. Small, rounded mitochondria are present throughout the cytoplasm and slender strands of rough ER accumulate around the nucleus (Fig. 113). A single developing Golgi body is found amongst the ER. Immediately after cell division the secretory cells grow towards the duct lumen, displacing the epithelial cells, while electron-dense particles (diam. 0.02 - 0.06µm), probably glycogen, accumulate above the nucleus (Fig. 114). At this stage these granular aggregates are the only means of distinguishing between secretory and epithelial cells.

ii) The accumulation phase

The secretory cells enlarge and the nucleus now lies at the base of the cell, where there is rapid multiplication of organelles in the surrounding cytoplasm (Fig. 115). The rough ER becomes filled with a pale, amorphous secretion and areas of parallel cisternae, viewed in transverse section, give the appearance of ribosome-coated vesicles. Large cup-shaped Golgi-bodies with stacks of up to 15 cisternae are common (Fig. 116). At this stage the

inner cisternae contain small quantities of an electron-dense, granular material and one or two vesicles (max. diam. 0.25μm), with similar contents, are seen in the neighbouring cytoplasm. Pale-staining vesicles, possibly arising directly from the ER, fuse to form large, pale, secretory granules. As these flow towards the apex of the cell the secretion condenses and an electron-dense core develops (Figs. 115 & 117).

A few small, rounded mitochondria are scattered throughout the cell and polysomes give the cytoplasm a granular appearance. The glycogen stores are dispersed, reduced, or absent.

The cells are pyramidal in shape, tapering towards their apices, and only a small area opens onto the duct lumen. The apical membrane is densely populated with short microvilli (Fig. 118). Small vesicles (diam. 0.05 - 0.08μm) are associated with the apical membrane and may represent pinocytotic activity. Large numbers of longitudinally orientated microtubules, approximately 20nm in diameter and at least 2μm long, are present in the apex of the cell (Figs. 117 & 118). Desmosomes are present at the luminal surface and zonulae adhaerentes occur at regular intervals along the longitudinal membranes (Fig. 119). Occasional hemidesmosomes may be seen along the basal membranes.

iii) The secretion phase

As the albumen gland matures ER and Golgi body activity increases. The rough ER becomes very extensive and its convoluted cisternae fill the basal cytoplasm (Fig. 120). Tubular extensions of smooth ER, or smooth vesicles, bud out from the rough ER nearest the Golgi apparatus, and many electron-opaque vesicles (av. diam. 0.06μm) accumulate near the inner aspect of the Golgi stack (Fig. 121). The Golgi bodies now display a distinct polarity, and a dense, granular secretion accumulates in the inner cisternae. These cisternae become very distorted and give rise to large irregularly-shaped vesicles.

The vesicles fuse and form large granules which flow towards the apex of the cell. Mature granules may exceed 11μm in diameter and a denser spot (diam. 1 - 2μm) usually develops near the periphery (Fig. 122) A pale, flocculent material tends to accumulate around the granules at the apex of the cell. It does not appear to be produced by either the rough ER or the Golgi apparatus.

As the secretion accumulates the secretory cells expand and their swollen bases fill the sub-epithelial space. Their apices open into the ductule at regular intervals along its length and the apical membrane is

fringed with numerous microvilli (Fig. 122). The epithelial cells lining the ductule are generally columnar, becoming distorted when the apices of the large secretory cells expand (Fig. 123). An elongated, basal nucleus occupies approximately 2/3 of the cell. It contains a central nucleolus but relatively little heterochromatin. The Golgi apparatus is small and consists of only one or two stacks of 5 to 7 cisternae (Fig. 124). Small, clear vesicles (diam. 0.04 - 0.08μm) lie around the outer, convex surface. The rough ER is insignificant and only a few slender cisternae are seen. Free polysomes are dispersed throughout the cytoplasm and small, rounded mitochondria lie near the cell apex. Clear vesicles (diam. 0.05μm), possibly pinocytotic, are frequently seen at the apical membrane. Larger vesicles (max. diam. 0.02μm) and MVBs may also be present. The apical membrane is densely ciliated and bears numerous microvilli. Belt desmosomes (0.2μm thick) surround the cell apices and zonulae adhaerentes are present at regular intervals along the longitudinal membranes giving them a characteristic pattern of alternating light and dark bands (Fig. 124).

During the secretory phase albumen gland secretion, together with cell debris, is often seen in the lumen of the ductules and collecting ducts (Figs.123 & 125). The epithelial cells lining the main collecting ducts show little specialization or modification. They

remain cuboidal and have large central nuclei containing only small quantities of chromatin (Fig. 125). A small Golgi body lies above the nucleus, and the stacks of 5 to 6 slightly concave cisternae are surrounded by clear vesicles (diam. 0.05 - 0.1μm). Strands of rough ER surround the nucleus and free ribosomes are scattered throughout the cytoplasm. Cylindrical mitochondria tend to be concentrated near the cell apex (Fig. 126). Again, the apical membrane is ciliated with many, stubby microvilli. Desmosomes form continuous bands around the apices of the cells. A typical belt desmosome, with a dense plaque and radiating filaments, is seen for the first 0.2μm, and beneath this a septate desmosome, with regular cross-striations, extends for a further 0.4-0.6μm (Fig. 127). In addition, hemidesmosomes are present along the basal membrane. Both longitudinal and basal membranes show some degree of folding.

iv) The connective tissue sheath

The secretory cells of the albumen gland are bound together by a thin layer of connective tissue to form discrete acini (Fig. 128). The major cell components of this connective tissue layer are the pigment cells, which are dispersed throughout the collagen matrix. Small compact groups of muscle cells and axons run alongside the ductules for most of their length (Fig. 129). Peripheral neurons are rarely seen.

ULTRASTRUCTURE OF THE CARREFOUR REGION IN A.HORTENSIS

1. The mature hermaphrodite duct

As in D.reticulatum the slender regions of the hermaphrodite duct are heavily ciliated, and as the duct expands to form the seminal vesicle these ciliated cells are reduced to a continuous band one quarter to one third of the duct's total circumference (Fig. 6). Unciliated cells line the remaining portion of the duct.

The seminal vesicle

i) The ciliated cells

The ciliated epithelial cells are columnar with rounded, central nuclei containing a few small clumps of chromatin (Figs. 130 & 131). The Golgi apparatus lies near the nucleus, but it is insignificant and seldom seen. Small, clear vesicles (diam. 0.05μm) and larger, denser vesicles (max. diam. 0.4μm) are occasionally seen within the cytoplasm, but multivesicular bodies are rare (Fig. 132). In older animals clear intercellular vacuoles develop, their size and frequency increasing with age (Figs. 130 & 131).

The cytoplasm is pale-staining due to the presence of glycogen, but in contrast to that of D.reticulatum it is dispersed throughout the cell and does not form homogeneous aggregates around the nucleus. This, together with the large numbers of polysomes, gives the cell a very granular appearance.

Longitudinally-orientated microtubules and microfilaments are present at the periphery of the cell, and cylindrical mitochondria are concentrated near the apex (Fig. 132). The apical membrane bears numerous, long microvilli and regularly-spaced cilia. The ciliary rootlets extend into the cytoplasm for 2µm and paired, lateral "feet" are associated with the basal bodies. Belt desmosomes and zonulae adhaerentes are present at the luminal surface forming junctional complexes approximately 1µm thick.

ii) The unciliated cells

As in D.reticulatum the shape of the unciliated epithelial cells changes as the duct fills with sperm. Newly differentiated cells are columnar (cell height 13 - 20µm) (Figs. 130 & 133), but as the sperm volume increases they stretch and become cuboidal (cell height 6 - 10µm) (Fig. 134), which results in a four-fold increase in duct diameter. These cells do not appear to become squamous.

The large, central nuclei are rich in chromatin, having a characteristic speckled appearance. The Golgi apparatus is small, consisting of compact stacks of 5 to 6 concave cisternae. Clear vesicles (diam 0.05μm) lie close to the inner surface. Larger vesicles with clear contents (max. diam. 0.2μm), electron-dense vesicles and multivesicular bodies accumulate sub-apically, and small chains of vesicles, probably pinocytotic, are present at the apex (Fig. 135). In older animals vacuoles develop, both inter- and intra-cellularly (Fig. 134).

Electron-dense bodies are found in many cells (Figs. 130 & 133 - 135) and these appear to be the partially-digested remnants of phagocytosed sperm (Fig. 136). A few small mitochondria and free polysomes are scattered throughout the cytoplasm but no glycogen is present.

The apical membrane bears numerous microvilli, but their distribution over the cell surface is irregular and some areas are heavily populated while others are completely smooth (Fig. 135). Small belt desmosomes (0.1μm thick) surround the apex of each cell and beyond this, a zonula adhaerens extends for a further 1.5μm. In addition, belt desmosomes (0.15μm thick) are present at the bases of the cells (Fig. 137).

The slender coiled region of the hemaphrodite duct

The cells of this region are similar to the ciliated cells of the seminal vesicle (Fig. 138) but as the duct approaches the carrefour loop the glycogen gradually disappears from the cytoplasm.

2. The mature carrefour loop

The looped portion of the carrefour is lined by highly interdigitating columnar epithelial cells with prominent basal nuclei containing irregular clumps of chromatin (Fig. 139).

The rough ER is insignificant and only a few slender cisternae are seen in the perinuclear cytoplasm. A single Golgi stack of 4 to 6 cisternae lies above the nucleus and this is surrounded by a cluster of pale-staining vesicles (diam. 0.05 - 0.2μm) (Fig. 140). The smaller vesicles may fuse, giving rise to the larger variety.

An heterogeneous collection of vesicles accumulates near the cell apex (Fig. 141). Many small, clear vesicles (av. diam. 0.1μm) are present throughout the cytoplasm and may form short chains, associated with the apical membrane. Multivesicular bodies are frequently seen at the apex. Larger vesicles (max. diam. 0.5μm)

accumulate sub-apically. Their contents range from clear to electron-dense and they appear to condense with age. These may be lysosomal, and in older animals (stages E to H) secondary lysosomes and residual bodies are readily identified (Fig. 142).

Longitudinally-orientated microtubules lie around the periphery of the cell (Fig. 142) and polysomes are scattered throughout the cytoplasm, giving it a granular appearance. Cylindrical mitochondria are concentrated at the cell apex. The apical membrane bears both microvilli and cilia, the long ciliary rootlets extending 3µm into the cytoplasm. Belt desmosomes (0.3µm thick) surround the apex of each cell and zonulae adhaerentes are present at regular intervals along the longitudinal membranes. Hemidesmosomes are frequently seen at the cell bases. The basal membrane is generally folded and intimately related to the underlying nerve plexus (Fig. 143).

3. The carrefour gland

The development of the carrefour gland is similar to that of D.reticulatum. Proliferation and differentiation of the mesenchyme cells in the connective tissue sheath begins during the early C stage. As the secretory cells mature they grow towards

the duct lumen, pushing in between the epithelial cells and distorting the epithelium (Fig. 144).

The carrefour gland cells are pear-shaped with irregular, basal nuclei (Fig. 145). Each nucleus contains a prominent nucleolus and small clumps of chromatin line the nuclear envelope. The cytoplasm is filled by an extensive Golgi complex and the swollen cisternae of rough ER (Fig. 146). Both organelles are involved in the production of secretory granules but their relationship is not clear. The Golgi complex consists of numerous concave stacks of 8 to 16 cisternae. The innermost cisternae swell and form secretory vesicles, approximately 0.1μm in diameter. These vesicles fuse to form large granules (diam. 1-2μm) which accumulate at the apex of the cell. The cisternae of the rough ER are filled with rod-like granules similar to those observed for D.reticulatum (Fig. 146). Vesicles bud off from the cisternae and appear to fuse directly with the large developing granules.

As the secretion matures it becomes less dense and the granules merge together to form an homogeneous mass at the apex of the cell (Fig. 147). The apical membrane bears microvilli (Fig. 148), and when this ruptures the secretion flows into the duct lumen.

4. The carrefour at the junction of the albumen gland ducts and common duct

Here, the epithelium becomes very distorted as large clear vacuolated areas appear between the cells (Fig. 149). The epithelial cells are reduced to thin strands of cytoplasm connecting the ciliated apices to the basal nuclei.

The nuclei become elongated, lying parallel to the longitudinal axes of the cells (Fig. 150). They contain a prominent, central nucleolus which is surrounded by smaller clumps of chromatin. Large aggregates of glycogen are present beneath the nucleus and at the apex of the cell. Only a narrow strip of cytoplasm (approx. 4µm wide) remains at the apex (Fig. 151). This contains a high concentration of vesicles (diam. 0.05 - 0.5µm) and small cylindrical mitochondria. The smaller vesicles normally have clear contents, while the larger are electron dense.

The apical membrane is ciliated and bears a fringe of microvilli. Belt desmosomes (0.3µm thick) are present at the apex of the cell, and immediately beneath, septate desmosomes extend for a further 1µm. This forms a strong junctional complex which limits the intercellular vacuolation. The basal membrane is very irregular but there is still an intimate relationship

with the underlying nerve plexus, and, in this region of the carrefour, many sensory neurons extend through the epithelium (Figs. 150 & 152).

5. The albumen gland

The albumen gland of A.hortensis is similar to that of D.reticulatum and follows the same pattern of development.

By the end of the B stage the albumen gland consists of two simple sacs which open into the carrefour via short ducts (Fig. 39). Maturation proceeds throughout the C stage with proliferation, accumulation and secretory phases. The ultrastructure of the mature gland appears identical to that of D.reticulatum (Figs. 153 & 154).

6. The connective tissue sheath

The connective tissue surrounding the hermaphrodite duct is similar to that described for D.reticulatum. The basement membrane (50nm thick) is surrounded by a layer of collagen (0.2 - 0.4µm thick), a band of circular muscle and then an outer layer of longitudinally orientated muscle fibres (Figs. 130, 134 & 137). Pigment cells are occasionally found between the muscle layers and also at the periphery of the

connective tissue sheath. A few small nerves of only 20 to 30 axons may also be present and a single large nerve containing between 60 to 70 axons runs alongside the duct. The extracellular matrix is composed of loose bundles of collagen in an amorphous ground substance.

Large irregularly-shaped pore cells are occasionally found around the hermaphrodite duct and are easily recognisable, having the characteristic surface slits (Figs. 155 & 156). A well developed rER surrounds the nucleus and the swollen cisternae are filled with a pale-staining, flocculent material. In many animals large accumulations of this material have displaced the cytoplasm to the periphery of the cell where the organelles become highly concentrated. The Golgi apparatus is inconspicuous, but is usually surrounded by many clear vesicles (diam. 0.05 - 1.5μm). Other, denser vesicles, with unit membranes, are common throughout the peripheral cytoplasm, but their origin is unknown. Large numbers of secondary lysosomes are seen in the pore cells of older animals.

Connective tissue completely ensheaths the carrefour and its associated glands. Fibroblasts are scattered throughout the loose collagen matrix and, as in D.reticulatum , there are many muscle fibres and an extensive nerve plexus (Fig. 157).

The connective tissue surrounding the descending arm of the carrefour loop is highly organized (Fig. 158). A continuous, thin band of circular muscle lies beneath the basement membrane, and completely surrounds the duct. A few small nerves are present between this and a second circular muscle layer, and at the periphery there is an outer layer of nerves, neurons (Fig. 157a) and occasional pigment cells.

As the duct loops round, this organization is lost, and the connective tissue sheath becomes much thinner (Fig. 159). The circular muscle layers are replaced by a mixture of longitudinally and obliquely orientated fibres and the nervous material develops an intimate relationship with the epithelial cells, with many nerves lying in grooves at frequent intervals along the basal membranes of the cells (Figs.157b & 160).

In the region of the carrefour gland the connective tissue stretches to surround the sub-epithelial secretory cells (Fig. 161), although the nerve plexus remains concentrated close to the duct epithelium (Figs. 157c & 162). The secretory cells become constricted as they near the epithelium and their slender necks appear to be ringed by small nerves (Figs. 162 & 163).

As the duct approaches the junction with the albumen gland ducts and common duct the connective tissue becomes very thick and is densely packed with muscle, nerve and occasional pigment cells (Fig. 164). The muscle is both longitudinally and obliquely orientated, frequently establishing close contacts with neighbouring nerves, where neuromuscular junctions may be seen (Fig. 165). Many nerves lie within grooves along the bases of the epithelial cells (Fig. 166) and sensory receptor neurons extend through the epithelium (Figs. 149 & 152). These specialized cells have obvious polarity with their apices at the luminal surface (Fig. 152) and their bases maintaining contact with the sub-epithelial nerve plexus (Fig. 167). The apical membrane bears numerous microvilli but only a single cilium. In all other respects these elongated sensory receptor neurons are similar to sub-epithelial neurons.

The acini of the albumen gland are surrounded by a thin layer of connective tissue. Large numbers of pigment cells are dispersed throughout the collagen matrix while muscle fibres and nerves tend to be concentrated around the ductule epithelium.

At the ultrastructural level, the nerve, muscle and pigment cells (Figs. 164 & 168) are similar to those of D.reticulatum (Figs. 88 & 90). The principal

difference lies in the appearance of the nerves. They tend to be smaller in A.hortensis, the largest containing up to 70 axons, and the axons are more loosely packed together so that even in mature animals they have a circular cross-section (Fig. 169).

DISCUSSION

The hermaphrodite duct

The morphology and ultrastructure of the hermaphrodite ducts in D.reticulatum and A.hortensis are typical of stylommatophoran pulmonates, having a narrow posterior region, formed by the confluence of efferent ductules from the gonad, a seminal vesicle (= vesicula seminalis) and a slender anterior region which enters the carrefour. The seminal vesicle is normally a simple duct capable of considerable expansion, although in the Streptaxidae sperm storage is restricted to a single sac-like region of the hermaphrodite duct (Berry,1963; Visser, 1973). In contrast, the seminal vesicles of basommatophoran pulmonates are frequently very elaborate structures with many lateral diverticula (Berry, 1977; Duncan, 1960b).

Earlier work on the hermaphrodite duct of D.reticulatum has shown that prior to maturation, the whole duct is uniformly lined by sparsely ciliated,

glycogen-rich, epithelial cells (Hogg and Wijdenes, 1979). Only when the duct starts to fill with sperm do the cells differentiate into ciliated and non-ciliated cell types. The presence of a continuous band, or ridge, of cilia has been observed in other pulmonates (Duncan, 1960b; Berry, 1977) and appears to be a consistant feature.

Expansion of the seminal vesicle is achieved by the unciliated epithelial cells stretching until they become cuboidal or squamous, while the ciliated cells, which experience the same stresses, remain columnar. Maintenance of cell height in just the one cell type must be due to the internal skeleton of microtubules and microfibrils. This is completely absent in the readily deformed, unciliated cells.

As the duct fills with sperm both cell types experience increasing mechanical stress and the intercellular attachments must withstand these pressures. Junctional complexes are well developed, particularly around the cell apices, and hemidesmosomes provide firm attachment to the underlying connective tissue via the basement membrane. Long, ciliary rootlets with prominant, lateral, basal "feet" are another indication that the cells are subjected to considerable external pressure (Dustin, 1978). They

provide stability for the cilia which must continue to function when the duct is fully distended.

Some vesicles in the cell apex may be secretory, releasing their products by exocytosis. In Lymnaea stagnalis secretory cells in the hermaphrodite duct produce a non-glycogen polysaccharide (Holm, 1946). It has been suggested that this is necessary for maintenance of the stored sperm, but since the spermatozoa appear to be inactive at this stage, their nutrient requirement is low. Secretion of a large molecular weight protein may raise the viscosity of the surrounding fluid beyond the point where sperm can swim. This has been reported in the caudal epididymus of rats where the glycoprotein responsible has been termed "immobilin" (Usselman and Cone, 1983). A third possibility is that massive release of secretion at copulation fluidizes the sperm, facilitating its movement towards the carrefour and enabling the cilia to act more effectively (Runham, 1984). In both D.reticulatum and A.hortensis , however, there is no evidence that large amounts of secretion accumulate and there is a very low level of exocytosis - if any. Thus, the contribution of secretion to sperm maintenance, sperm immobilization or sperm fluidization must be minimal.

Retrieval of the vesicle membrane following exocytosis could produce the short chains of pinocytotic vesicles seen near the apical membrane. Continuous low-level pinocytosis would remove small volumes of fluid from the duct lumen, and the appearance of large, intercellular vacuoles, in older animals, suggests that there is steady accumulation. This may be necessary for controlling the fluidity of the sperm mass.

The unciliated cells regularly phagocytose sperm. This has been reported in Oxychilus cellarius (Rigby, 1963), Helix pomatia (Breucker, 1964; Lind, 1973), Biomphalaria glabrata (Jong-Brink, 1969), Achatina fulica (Breckenridge and Fallil, 1973) and Littorina scutula (Buckland-Nicks and Chia, 1976). Lind (1973) observed that in H.pomatia sperm was expelled from the hermaphrodite duct at times other than copulation. This sperm passes directly into the bursa copulatrix and it appears that while abnormal spermatozoa are removed by phagocytosis within the hermaphrodite duct, large numbers of aging sperm are digested in the bursa. Occasionally, sperm was seen in the reproductive tracts of non-copulatory D.reticulatum and A.hortensis and a similar method of sperm removal may operate in these two species. The presence of multivesicular bodies and large phagocytotic vacuoles suggests that some of the small cytoplasmic vesicles are in fact primary lysosomes originating from the transitional ER. Thus, the

principal role of the unciliated cells appears to be one of absorption and lysosomal destruction of sperm, and this agrees with the conclusions of Jong-Brink (1969) who first cast doubt on the importance of secretion by these, so-called, "secretory" cells.

The carrefour diverticula, loop or "talon"

A small ovoid swelling or slender elongated appendage arises from the carrefour of most gastropod molluscs. In Acteon tornatilis, an opisthobranch which appears to bridge the evolutionary gap between the prosobranchs and primitive pulmonates (Duncan, 1960a), a slender duct terminates in a small, rounded saccule known as the seminal receptacle (Fretter, 1946). In pulmonates this is replaced by a swollen chamber or diverticulum which lacks the long duct, and is generally believed to be the site of fertilization. For this reason it has been termed the fertilization pouch.

Amongst the Basommatophora, the four families belonging to the Hygrophila have received most attention (Crabb, 1927; Holm, 1946; Abdel-Malek, 1954a, 1954b; Alaphilippe, 1959; Alaphilippe and Régondaud, 1959; Duncan, 1958, 1960a, 1960b; Walter, 1968; Jong-Brink, 1969). Here, the fertilization pouch is a single or multi-lobed swelling near the junction with the albumen gland, and ciliated channels direct the gametes to

either the male or female ducts of the anterior reproductive tract. In the Physidae, where the albumen gland duct is absent, the ova pass through the albumen gland itself before being directed into the oviduct. In the Planorbidae, Ancylidae and Lymnaeidae, however, albumen is added to the fertilized ova as soon as they leave this swollen, pouched area.

The Stylommatophora normally have at least two diverticula associated with the carrefour and their combined structure gives rise to a small appendage, bound in connective tissue, which french workers have termed the "talon" or "claw". Although superficially these structures show considerable variation in complexity, their basic morphology is surprisingly uniform. The hermaphrodite duct opens into a large, flattened saccule which wraps around one or more central diverticula giving it a characteristic crescent-shape in transverse section.

The variety of form arises from branching or pouching of either the central diverticulum or, more rarely, the outer saccule (Fig. 170). The basic arrangement is seen in Sphaerospira fraseri, a member of the Camaenidae and primitive to the Helicidae (Bishop, 1978), Bradybaena fruiticum (Shileyko and Shileyko, 1975), Oxychilus (Rigby, 1963; Flasar, 1967) and throughout the Limacidae, where both the pouch and

single diverticulum are simple structures (Fig. 170a). Branching of the diverticulum into 3 to 5 slender saccules of uneven length, gives the characteristic helicid carrefour (Fig. 170b), e.g. Helix pomatia (Lind, 1973) and Theba pisana (Noyce, 1973), but the greatest elaboration is seen in the Bulimulidae (Mol, 1971) and Oreohelicidae (Shileyko and Shileyko, 1975), where dichotomous branching of the diverticulum produces tubules of variable lengths and number (Fig. 170c), e.g. 8 in Pellicula depressa and 34 in Drymaeus papyraceus. In Succinea putris two separate diverticula open into the flattened sacccule (Rigby, 1965) (Fig. 170d). This is an unusual arrangement and has only been recorded in the Succineidae. Interestingly, in one species, S.pfeifferi the two diverticula are united basally, and this bifid structure falls more readily into the general pattern of carrefour morphology (Fig. 170e). Elaborations in the saccule are seldom seen, but combinations of either a simple or branched diverticulum, with a pouched saccule (Figs. 170f& g) have been reported in Trichia hispida and Triodopsis multilineata, respectively (Shileyko and Shileyko, 1975).

Exceptions to this basic arrangement have been observed in several species of slug. Lüsis (1961) first reported the absence of separate diverticula in Arion ater rufus , where the talon is formed by the

anterior slender region of the hermaphrodite duct elongating and describing a simple loop before entering the carrefour (Fig 170h). This study has shown that other members of the Arionidae, e.g. A.hortensis , A.intermedius, A.fasciatus and A.ater ater, have similar morphology, and the same structure has been reported in the closely related American slugs, Philomycus bilineatus, (Ikeda, 1937) and P.carolinianus (Kugler, 1965). In an African slug, Aillya camerunensis, the talon is absent (Mol,1978). The hermaphrodite duct forms no looped structure and passes directly into the carrefour (Fig. 170i). The anterior region of this duct, however, appears to be functionally comparable to the carrefour loop of arionid and philomycid slugs.

An anomaly arises in the classification of limacid and arionid carrefours. Els (1974) reports that the carrefour of Milax gagates takes the form of a hairpin loop. This is the characteristic structure seen throughout the Arionidae, while other Milax species, e.g. M.sowerbyi and M.budapestensis, have the two diverticula common to the limacid slugs. Unfortunately M. gagates was not available for examination during this study and it has not been possible to confirm Els' findings. The external appearance described by Taylor (1907) and Quick (1960) both indicate that the talon has

an ovoid rather than elongated form, which questions the identification of the South African Milax gagates.

Despite the obvious difference in morphology, the talons of D.reticulatum and A.hortensis have similar ultrastructure. Both are capable of expansion and show many of the adaptations described in the hermaphrodite duct, e.g. an internal skeleton of microtubules, highly developed junctional complexes and long, ciliary rootlets, although the lateral 'feet' appear to be absent. In addition, these cells are highly tesselated which further reduces the effect of shearing.

The appearance of the ER-Golgi body complex and the large number of polysomes suggest protein synthesis, and exocytotic release of this secretion could account for the accumulation of pinocytotic vesicles seen near the cell apex. These vesicles may contribute to the formation of MVBs and, as in the hermaphrodite duct, lysosomes accumulate with age. Thus, some of the vesicles arising from the ER-Golgi apparatus are primary lysosomes.

Low level release of secretion and/or continuous removal of luminal fluid by pinocytosis has no obvious function in the talon. This activity may increase prior to ovulation and oviposition, while phagocytosis of

abnormal or excess gametes, following copulation or egg-laying, may give rise to the secondary lysosomes.

The epithelium of the talon is densely ciliated and Rigby (1963) referred to the flattened saccule in Oxychilus cellarius as the ciliated hood. This terminology is frequently used by other authors, including Sirgel (1973), Noyce (1973) and Stears (1974) and tends to imply that the associated diverticulum is unciliated. This, however, is not always the case and most reports describe long cilia extending into the lumina of both diverticula (Mol, 1971; Lind, 1973; Sirgel, 1973; Stears, 1974). For clarity, it is perhaps advisable to avoid this descriptive term. The unciliated regions in the flattened saccule of D.reticulatum do not appear to be functionally significant.

Flask-shaped mucous cells have been observed in the connective tissue surrounding the talon of many pulmonates, opening through the epithelium of the flattened pouch, e.g. Deroceras caruanae (Sirgel, 1973) and Limax valentianus (Stears, 1974). Since the oocytes accumulate here during oviposition, the release of mucus may be important at this time. In D.reticulatum the frequency of mucous cells varied between animals and it is possible that their number increases with age. No mucocytes were observed around the carrefour of

A.hortensis, and a similar report on the carrefour loop of Milax gagates (Els, 1974) suggests that mucus secretion may be reduced or absent in this type of talon.

The carrefour gland

There are many reports of flask-shaped gland cells concentrated around the carrefour. They extend from the junction with the talon to the proximal region of the male groove and have been confused with both mucocytes (Kugler, 1965) and oviducal gland cells (Visser, 1973). However, their differential staining (Sirgel, 1973) and their ultrastructural appearance indicates that these form a separate gland. Previous authors have named this region mucous gland I of the common duct (Visser, 1973) or the nidamental gland (Els, 1974). Visser's "mucous" gland failed to stain with conventional Azan techniques and the mucus content is therefore doubtful. Nidamental gland is a confusing term and can refer to all the secretory cells which contribute to the formation of eggs (Thompson and Bebbington, 1969). In the present study therefore, it was decided to call this the carrefour gland, indicating its position but leaving its function open to discussion.

Throughout this investigation the carrefour glands of all mature animals were observed to release copious, clear secretion into the lumen of the duct. This secretion is a product of the rough ER and Golgi apparatus, suggesting that it is a glycoprotein. It is alcian blue- and P.A.S.- negative and therefore, does not appear to be a mucin. Slight colouration with bromophenol blue, however, indicates a proteinaceous component. Since the gland appears to secrete continuously its function is not directly related to either oviposition or copulatory activity. Runham (1984) suggests that the secretion from the albumen gland requires dilution before it is added to the oocytes, and this gland may be involved. Alternatively, continous secretion may simply lubricate the reproductive tract.

The junction of the albumen gland ducts and common duct in A. hortensis

When the carrefour of A.hortensis was examined in the electron microscope a highly vacuolated region appeared at the junction of the albumen gland ducts and common duct. This cannot be identified in sections prepared for light microscopy although the "empty" nature of the tissue suggests that it should be recognized easily. This region has not been reported in other related species nor was it observed in

D.reticulatum, and in the light of these observations it is tempting to ignore this area believing it to be an artifact.

Distortion of the unit membranes and cytoplasmic organelles is usually a good indication of poor tissue preservation, but in this case they appear perfectly normal. In addition, the cytoplasm of the epithelial cells contains large aggregates of glycogen, which is absent in the adjacent non-vacuolated regions, while the underlying nerve plexus extends further into the epithelium and many sensory neurons are present. These features indicate that this area is a highly significant region of the carrefour. Its sensory function cannot be ignored, and it possibly detects the passage of gametes, while the vacuoles suggest that it is involved in pumping fluids across the epithelium.

The albumen gland

In the majority of pulmonates the albumen gland consists of a single large lobe which opens into the carrefour near the junction with the common duct (e.g. Rigby, 1963; Lind, 1973), but in the two slugs studied and in members of closely related slug species e.g. Deroceras caruanae (Sirgel, 1973), Milax gagates (Els, 1974) and Limax valentianus (Stears, 1974) the albumen

gland is a bilobed structure with two separate ducts. The significance of this, however, is not clear.

The albumen gland has been the subject of numerous histological and ultrastructural studies, including those of Baecker (1932), Nieland and Goudsmit (1969) and Jong-Brink (1969), and it appears that there is little variation in the basic structure. It is a compound tubular gland with large secretory cells, which Visser (1973) confirms are of mesenchymal origin.

In D. reticulatum and A. hortensis the nature of the secretion alters with age. At the onset of secretory activity numerous large, pale-staining vesicles are produced, their fine particulate contents resembling that seen in the swollen rough ER. The secretion produced by the ER may be further processed in the Golgi cisternae or it may transform directly into these early pale vesicles.

The Golgi apparatus is only just beginning to synthesize the electron-dense material that fills mature secretory granules. Possibly, this initial low ratio of dense to pale secretion results in the small, dense core, seen only in the condensing vesicles of immature glands.

As Golgi synthesis increases the secretory granules become uniformly electron-dense and at high magnifications small, denser particles (diam. 0.02 μm) can be identified within the homogeneous matrix. In addition, large numbers of similarly-sized particles are present throughout the cytoplasm. In Helix pomatia the particles within the secretory granules are galactogen, but since ribosomes, beta-glycogen and galactogen are all approximately the same size it is not possible to identify the cytoplasmic particles with any certainty (Nieland and Goudsmit, 1969).

The pale, flocculent material which accumulates around the mature secretory granules in D.reticulatum and A. hortensis is not reported by either Nieland and Goudsmit (1969) or Jong-Brink (1969, 1973). Occasionally, glycogen is removed during the preparation of tissue for electron microscopy, and these empty-looking spaces may indicate the position of dissolved glycogen. However, it is generally recognized that the gland grows at the expense of glucose or glycogen (Tompa, 1984), and large stocks of carbohydrate are therefore unlikely to be present at this time.

The albumen gland fluctuates in size throughout the reproductive cycle. Runham and Laryea (1968) demonstrated that it is largest just before egg-laying commences, up to 6% of the total body weight in

D.reticulatum, while in those animals which have just completed laying it is significantly smaller. The capacity of the albumen gland probably determines the number of eggs laid at any one time.

The albumen or perivitelline fluid provides nutrient and probably water for the developing embryo, supplementing the yolk supplies of the ova. Galactogen is found only in the albumen gland and the digestive tract and hepatic lobe of embryonic or newly-hatched slugs (Carrick, 1938). Consequently, it appears to be most important for embryonic development, while its high molecular weight maintains the osmolarity of the egg. Bayne (1969) demonstrated that when the humidity falls the eggs of D.reticulatum preferentially lose water from the albumen layer, so that the embryo is affected less severely, but subsequent rehydration is then rapid due to the albumen's great affinity with water.

The connective tissue sheath

The connective tissue surrounding the hermaphrodite duct is highly muscular and the layers of circular - and longitudinally-orientated fibres permit considerable expansion, while peristaltic contractions at copulation would assist in the bulk movement of sperm.

In A.hortensis the layers of circular muscle continue into the looped region of the carrefour, and the descending limb may also be capable of peristalsis. As the duct loops around, however, the fibres become randomly-orientated. This coincides with an increase in the amount of nervous tissue and ramification of the axons precludes the presence of organized muscle layers. It will be shown later that the ascending limb of the carrefour loop, with its extensive nerve plexus, temporarily stores the oocytes during egg-laying, while the descending limb is simply a continuation of the hermaphrodite duct.

In both D.reticulatum and A.hortensis there were no identifiable sphincters for controlling movement of gametes into and out of the carrefour.

Previous descriptions of molluscan muscle cells (Nisbet and Plummer, 1968; Sminia, 1972a) suggest that there is little ultrastructural variation throughout the phylum. The thin filaments (diam. 5-10 nm) are believed to be actin and the thick ones (diam. 30-60 nm) mainly paramyosin (Sanchis and Zambrano, 1969). Sanchis and Zambrano also observed a third type of filament (diam. 12.5-17.5 nm) which is usually randomly-orientated amongst the rough ER near the nucleus. These were not seen in the present study.

The nerve net is a primitive feature in the evolution of the nervous system. It is first seen in the coelenterates, where impulses radiate out from the point of stimulation and result in either a general or localized response, depending upon the strength of the stimulus. This nerve plexus was retained in the ancestral molluscs, despite the development of a central nervous system. Subsequent concentration of the ganglia provided a coordinating centre for controlling complex behavioural patterns, and in the pulmonates the nerve nets have become very reduced and mediate local reflexes without involving the CNS.

An extensive sub-epithelial nerve plexus associated with the carrefour has been reported in a variety of basommatophoran and stylommatophoran snails (Brisson, 1983; Jong-Brink and Goldschmeding, 1983) and Brisson has demonstrated that it is principally an aminergic (synaptic) system with occasional, apparently peptidergic (neurosecretory) nerve fibres.

In Bulinus truncatus (Brisson, 1983) and Lymnaea stagnalis (Jong-Brink and Goldschmeding, 1983) sensory neurons are present in the albumen gland duct and the adjacent region of the carrefour. Similar cells were observed in the vacuolated epithelium of A.hortensis and they probably function as mechano- or chemo-receptors, mediating muscular, ciliary and glandular activity

according to the nature of the gametes passing through the duct.

Jong-Brink and Goldschmeding (1983) suggest that the sensory neurons play a major role in regulating albumen gland activity. If these neurons detect the passage of oocytes, they could conduct impluses, via the nerve net, to muscle cells surrounding the gland tubules. The resulting muscular contraction would then release an aliquot of secretion into the carrefour, and consequently each oocyte would be coated with approximately the same amount of vitellogenic fluid.

This is an attractive theory which could apply to A.hortensis, but to date, no sensory neurons have been located in D.reticulatum, despite a thorough search of the carrefour epithelium. This probably reflects their scarcity since it is unlikely that they are completely absent in this species, and only one or two are necessary to detect the presence of oocytes. Neuromuscular junctions are present in both Arion and Deroceras.

In addition to the obvious sensory neurons, stretch receptors may be present in the nerve net, which would be stimulated by expansion of the carrefour. These could act in exactly the same way, conducting nervous impulses to the target site.

Three types of vesicle are commonly found in molluscan neurons: (i) clear or electron-lucent, diam. 0.04 to 0.06μm, (ii) moderately-dense, diam. 0.05 to 0.1μm, and (iii) dense-cored, diam. 0.06 to 0.1μm (Gerschenfield, 1973; Roubos and Moorer-van Delft, 1979). Other granules and vesicles (diam. 0.05 - 0.25μm) may also be seen in the pre-synaptic element, together with coated vesicles from pinocytosis. These coated vesicles are the immediate consequence of exocytotic activity (Heuser et al. ,1979; Heuser and Reese, 1981).

True synapses are difficult to demonstrate without the use of a goniometer stage. Tilting the sections will usually resolve the dilemma of fuzzy membranes (Cobb and Pentraeth, 1977) and seven different types of synapses have now been described in L.stagnalis (Roubos and Moorer-van Delft, 1979). In the present study this facility was not available and no clear synaptic cleft could ever be identified. The position of a synapse, therefore, could only be assumed from the parallel orientation of the opposing membranes, the denser appearance of these membranes and the accumulation of pre-synaptic vesicles.

In both D.reticulatum and A.hortensis the pre-synaptic elements are packed with clear, and to a lesser extent, dense-cored vesicles. The concentration

of clear vesicles increases near the active site, but the dense-cored variety remain some distance away. Their size and arrangement both suggest that these are the type II synapses described by Roubos and Moorer-van Delft (1979).

Pore cells commonly occur in the connective tissue of gastropods and bivalves. Their characteristic surface morphology means that they are readily identified but their function is not so easily recognized. It has been suggested that they are involved in glycogen storage (Fernández, 1966), phagocytosis of particles in the haemolymph (Sminia, 1972), production of collagen and/or the collagen matrix (Plummer, 1966) and, finally, production of the blood pigments, haemocyanin and haemoglobulin (Sminia et al., 1972; Sminia and Boer, 1973).

The appearance of the pore cells in D.reticulatum and A.hortensis does not clarify the matter. Large accumulations of a granular material, resembling glycogen, are present in some cells but not in others. Sminia (1972) argued that in Lymnaea stagnalis vesicular connective tissue cells are the main storage site for glycogen and therefore the pore cells, containing only small deposits, are unlikely to duplicate this function. In the two slugs, however, no vesicular connective

tissue cells were observed and in their absence pore cells may assume the role of glycogen storage.

The presence of lysosomes and their accumulation with age suggests that in D.reticulatum and A.hortensis there is some uptake and degradation of material. Since true fixed macrophages appear to be absent in molluscan connective tissue, the pore cells may act as filters, removing waste particulate matter from the blood, and Sminia (1972) demonstrated that in L.stagnalis these cells are capable of selective phagocytosis.

There is no evidence to suggest that the pore cells of Deroceras or Arion synthesize collagen and the idea that collagen is produced by cells other than fibroblasts is not generally accepted. There is no conclusive proof for this theory and it would seem unlikely that the role of the fibroblast is duplicated.

It now appears that the production of blood pigment is the major function of pore cells. In Lymnaea, Biomphalaria, Planorbarius (Sminia et al., 1972; Sminia and Boer, 1973) and Limax (Reger, 1973) crystals are observed in the extensive rough ER and the cytoplasm may contain a lattice-like aggregate. Similarly, in A.hortensis (Skelding and Newell, 1975), where the rough ER is restricted to the peripheral cytoplasm by large stores of glycogen, the surface

cisternae are filled with haemocyanin. In the present study the ER was always confined to the periphery of the cell, having been displaced by either the glycogen-like material or lysosomes. Fine structural detail was obscured and the presence or absence of haemocyanin crystals could not be determined.

In conclusion, the pore cells in D.reticulatum and A.hortensis have three possible functions - glycogen storage, phagocytosis and synthesis of blood pigment, and their position at the periphery of the connective tissue sheath ensures that they are continually bathed in haemolymph fluid, facilitating their role in these last two processes. The function of the pores themselves remains unclear.

The pigment cells described in Arion ater rufus (Wondrak, 1969) and L.stagnalis (Sminia, 1972) are similar to those observed in D.reticulatum and A.hortensis. In A.ater, however, Wondrak reports that the Golgi apparatus is involved in the production of pigment granules, while in Lymnaea, Deroceras and A.hortensis the Golgi cisternae are insignificant, with clear contents. The rough ER on the other hand is swollen with secretion. The nature of the pigment is somewhat variable, and in A.ater rufus alone, three different pigments have been identified, porphyrins in the skin (Kennedy, 1959), lipofuscin surrounding the

gonad (Lüsis, 1962) and melanin throughout the connective tissue (Wondrak, 1969).

THE ANTERIOR REGION OF THE REPRODUCTIVE TRACT

The appearance of the anterior genital ducts contributes to the systematic classification of pulmonates and their comparative morphology is well described. However, to completely understand the functions of these ducts and their associated glands, more detailed information is necessary. Consequently, a comprehensive study of the anterior region of the reproductive tracts in both D.reticulatum and A. hortensis was carried out.

MORPHOLOGY AND MORPHOGENESIS OF THE ANTERIOR GENITAL DUCTS OF D. RETICULATUM AND A. HORTENSIS

1. Morphology of the mature penial mass and anterior genital ducts of D.reticulatum

The genital opening lies anteriorly, on the right hand side of the animal and may be partially covered by the mantle. It leads into a small atrium which immediately opens into a large, bulbous penial mass (Figs. 3a & 171). The penial mass is an elongated sac which tapers distally into a branching trifid appendage (Figs. 171 & 172). The morphology of the trifid appendage is very variable. In some animals it is a single diverticulum bearing three or four stubby side-branches (Fig 173a), while in others it is an

elegant, three-fingered structure with regularly spaced, rounded nodules over its surface (Figs. 173d & 174). Despite its sac-like appearance the internal structure of the penial mass is quite complex. One wall of the penial sac is greatly thickened and near the junction with the atrium it bears a solid, conical sarcobellum (Fig. 175). The sarcobellum may reach 5 to 6mm in length and its fine, tapering tip is frequently seen protruding into the atrium. The surface of the penial sac and sarcobellum is highly ridged giving it a characteristic crenellated appearance in transverse section (Fig. 176). A large gland is present at the base of the sarcobellum (Fig. 177). In older animals (Stages F to H) this becomes very extensive spreading up towards the tip of the sarcobellum and around to the opposing wall of the penial sac. This gland produces abundant secretion which stains positively with alcian blue and P.A.S. The penial mass is attached to the floor of the lung by the penial retractor muscle which inserts near the base of the trifid appendage (Fig. 171).

The vas deferens leaves the penial mass near the base of the trifid appendage (Fig. 175). It is a slender duct (diam. <50μm) (Fig. 178) and is approximately twice the length of the free oviduct. In contrast, the oviduct is much thicker (diam. 150 - 200μm) and also highly glandular (Fig. 178), the secretion staining positively with P.A.S. It passes out

of the genital atrium and extends for only a short distance before combining with the vas deferens to form the common duct (Fig. 179).

The bursa copulatrix leaves the atrium near its junction with the oviduct (Fig. 175). A short duct (Fig. 178) leads into a simple saccule which expands as it becomes filled with the waste products of reproductive activity (Fig. 179). This digestive sac is closely applied to the surface of the oviducal gland.

Two large arteries vascularize the anterior portion of the reproductive tract (Fig. 180). The penial artery, arising from the left cephalic artery, bifurcates as it approaches the penial mass and further branching of these smaller vessels results in an extensive "capillary" network radiating out over the entire surface. The anterior genital artery runs down along the common duct and later the free oviduct, terminating at the junction with the penial mass. A side branch of this artery vascularizes the bursa copulatrix.

Innervation of this area is achieved by the penial nerve and the third cutaneous pedal nerve (Fig. 181).

2. Morphogenesis of the penial mass and anterior genital ducts of D.reticulatum

In newly hatched animals the reproductive tract is a simple, undifferentiated tube (Fig. 182). Proliferation of a few cells near the genital opening results in a gradual increase in duct diameter and by the end of the A stage of development a small, ellipsoid swelling indicates the position of the adult genital atrium (Fig. 183).

During the early B stage the penial mass grows out to one side of this atrium (Fig. 184). Initially it is a simple flattened saccule but as it expands a solid ridge of cells develops along one wall (Figs. 184 & 185), and distally it tapers into an elongated, finger-like process. These elaborations of the penial sac correspond to the adult sarcobellum and trifid appendage respectively. Immediately behind the atrium a split develops within the reproductive tract, dividing it into the vas deferens and free oviduct. A slender, blind ending diverticulum buds off from the proximal end of the oviduct to form the rudimentary bursa copulatrix (Fig. 185). The separate male and female ducts extend for only a short distance before combining to form the common duct (Fig. 186).

The penial mass grows rapidly and morphogenesis is complete by the end of the B stage. The sarcobellum becomes a solid, conical structure which fills the penial sac (Fig. 187) and the trifid appendage develops many short, stubby side branches (Fig. 188). During this stage of development the surface of the sarcobellum and penial sac assumes the crenellated configuration characteristic of mature animals (Fig. 187).

Maturation proceeds throughout the C and D stages of development.

3. Morphology of the mature anterior genital ducts of A.hortensis

The genital opening lies anteriorly, on the right side of the animal. It leads into a lower and then an upper atrium (Figs. 189 & 190). A large, spongy gland surrounds the elliptical lower atrium (Figs. 190 & 191) and the secretion gives it a distinctive cream colour. In contrast the upper atrium is completely aglandular and its walls are thrown into deep folds (Figs. 190 & 192). This is the junction for the free oviduct, the separate male duct and the bursa copulatrix.

When the oviduct leaves the upper atrium it is surrounded by a thick layer of muscle and has a diameter of 0·8 to 1·0mm (Fig. 193). Approximately halfway

along its length it narrows abruptly and the diameter is reduced to only 200 to 260μm (Fig. 194). This fourfold decrease in duct diameter is due entirely to the loss of muscle, the diameter of the lumen remaining constant throughout its length. (diam. 100 - 180μm).

The male duct leaves the upper atrium at the same level as the oviduct but is approximately twice as long. The proximal third of this duct is known as the epiphallus. This is an expanded region whose inner surface bears numerous papillae (Figs. 195 & 196). At the junction with the upper atrium it extends into the atrial lumen as a glandular cone and the duct opens out at the apex of this (Fig. 197). At its distal end the epiphallus tapers into the vas deferens and the papillae gradually become smaller until they disappear altogether. The vas deferens is very slender (Fig. 198) and coils loosely before combining with the oviduct to form the common duct.

The bursa copulatrix extends from the base of the upper atrium. The duct of the bursa is short and the walls highly folded (Fig. 199). It opens out into a large, rounded sac which becomes filled with the waste products of reproduction (Fig. 200), and appears dark red in colour. A tough band of connective tissue anchors the tip of this digestive sac to the proximal end of the common duct.

The genital retractor muscle attaches the anterior genital ducts to the floor of the lung. Shortly after leaving the pallial region the muscle divides, one branch terminating at the upper atrium, the other half-way along the length of the oviduct (Fig. 189).

Two large arteries vascularize the anterior portion of the reproductive tract (Fig. 201). The penial artery, a branch of the cephalic artery, supplies the epiphallus and gives rise to numerous small vessels which radiate out over the whole surface. The anterior genital artery runs down along the common duct and later the free oviduct. On reaching the upper atrium it bifurcates, one branch running towards the lower atrium while the other vascularizes the bursa copulatrix.

4. Morphogenesis of the anterior genital ducts of A.hortensis

In newly hatched animals the reproductive tract is a simple undifferentiated tube (Fig. 202). Proliferation of cells near the genital opening results in a gradual increase in duct diameter and by the end of the A stage of development a small, ellipsoid swelling indicates the position of the adult atria (Fig. 203). This atrial area continues to expand (Fig. 204) and during the early B stage a split develops at its distal end which divides the reproductive tract into the vas

deferens and free oviduct (Fig. 205). A short, diverticulum buds off from the anterior end of the vas deferens, near its junction with the atrium. This is the rudimentary bursa copulatrix (Fig. 206). The separate male and female ducts extend for only a short distance before combining to form the common duct (Fig. 207).

Morphogenesis is complete by the end of the B stage. The vas deferens elongates rapidly and begins to form loose coils, while the bursa expands into a short, thick-walled saccule (Fig. 208).

Maturation proceeds throughout the C and D stages of development.

ULTRASTRUCTURE OF THE PENIAL MASS AND ANTERIOR GENITAL DUCTS IN D. RETICULATUM

1. The sarcobellum

The epithelium of the sarcobellum is folded into regular, longitudinally orientated ridges which give it a crenellated appearance in transverse section (Fig. 209).

At the apex of the sarcobellum these ridges are tall and slender (Figs. 210 & 211). The epithelial cells are cuboidal and have large, rounded, central nuclei,

each nucleus containing a single eccentric nucleolus and many irregular clumps of chromatin (Fig. 212). The perinuclear rough ER is filled with a pale-staining secretion. Vesicles (diam. 0.15 - 0.2μm) appear to bud from the swollen cisternae and accumulate above the nucleus in the vicinity of the Golgi complex (Fig. 213). The Golgi apparatus consists of several stacks of 5 to 6 concave cisternae, with many small clear vesicles (diam. 0.03μm) budding from the inner surface. These vesicles appear to interact with those produced by the rough ER and flow towards the apex of the cell. Other small vesicles (diam. 0.1μm) accumulate near the apical membrane, probably resulting from pinocytotic activity at the cell surface. They subsequently fuse forming larger, clear vacuoles (max. diam. 0.3μm) or MVBs (max. diam. 0.5μm). Secondary lysosomes may also be present.

Small cylindrical mitochondria are concentrated near the rough ER and also at the cell apex (Fig. 213). The apical membrane bears abundant microvilli and cilia, except for those cells at the bottom of the ridges where the cilia are absent (Fig. 214). In common with other tissues, these unciliated cells have fewer mitochondria at their apices.

Belt desmosomes (0.2μm thick) surround the apex of each cell (Fig. 213). Beyond this, clear spaces may develop between the cells, but their size is restricted

by small zonulae adhaerentes which are regularly dispersed along the longitudinal membranes. Numerous hemidesmosomes are present at the base of each cell.

As they approach the base of the sarcobellum the ridges become lower, broader and more widely spaced (Fig. 215). This is accompanied by small changes in the epithelium, the cells becoming columnar (Fig. 216) and highly interdigitating (Fig. 217).

The sarcobellum is a solid structure with a central blood sinus (Figs. 176 & 215), its cellular core composed of muscle, gland and connective tissue cells in a dense collagen matrix (Figs. 210 & 215). A layer of circular muscle follows the contours of the epithelium (Fig. 214) and the ridges tend to be filled by a network of longitudinally and obliquely orientated muscle fibres, to the exclusion of most other cells (Fig. 218). Beneath this, obliquely orientated fibres appear to spiral around the sarcobellum. This is seen most clearly at the apex, where the cellular core is reduced to just the sub-epithelial musculature (Fig.219). Nearer the base the sarcobellum is filled with developing gland cells, muscle and the very distinctive pore cells (Fig. 220). Nervous tissue appears to be absent.

Gland cells

Two types of gland cell are associated with the sarcobellum. They differentiate from unspecialized connective tissue cells during the C stage of development and their large, chromatin-rich nuclei are soon recognizable amongst the muscle and pore cells which make up the core of the sarcobellum (Fig. 221). Initially these gland cells appear identical but once synthesis of the secretory product begins two cell types can be distinguished.

Type I

The rough ER of the type I cells develops rapidly and the cytoplasm soon becomes packed with cisternae (Fig. 222). A pale staining secretion accumulates within the cisternae which swell, forming large rounded vesicles (Fig. 223). These vesicles appear to transform directly into secretory granules (max. diam. 1μm).

The secretory cells develop long, attenuated processes which push up between the epithelial cells (Fig. 224). They are packed with longitudinally orientated microtubules (Fig. 225) and the secretory granules flow along these processes towards the apex of the cell. Chains of small electron-dense vesicles

appear to connect the secretory granules (Fig. 225). Their function however is unknown. As the secretion matures the granules become electron-dense, and are released into the unciliated grooves on the surface of the sarcobellum (Fig. 226).

The Golgi apparatus of these cells lies near the nucleus but it is small and insignificant, consisting of 4 to 5 slender cisternae. There is no evidence that it is involved in the synthesis of the granules. Small, oval mitochondria are scattered throughout the cell (Fig. 227).

Type II

The type II gland cells differ from those of type I in two major respects. The rough ER is insignificant and only a few cisternae surround the nucleus, while the Golgi apparatus is highly developed and becomes very extensive as the cell matures (Fig. 228). Initially the Golgi stacks are compact consisting of 5 or 6 slender, concave cisternae (Fig. 229). Small, pale-staining vesicles (diam. 0.05μm) bud from the tips of the cisternae and flow towards the concave surface where they fuse with larger secretory vacuoles. As synthesis proceeds the cisternae become swollen and distorted and the secretion appears to contain fine, electron-dense particles or fibres (Fig. 230).

Eventually the whole cell becomes packed with this secretion. The tissue is then extremely difficult to section and is very unstable in the electron microscope.

As the secretion accumulates the cells grow towards the epithelium, pushing up between the epithelial cells so that their apices open onto the surface of the sarcobellum (Figs. 224 & 231).

The cytoplasm of these cells contains large numbers of polysomes. The mitochondria are less numerous than in the type I cells and tend to be more elongated.

When the secretory cells first differentiate the two cell types are in roughly equal numbers. Rapid multiplication of the type II gland cells results in a large homogeneous gland developing near the base of the sarcobellum. This spreads up towards the tip and around to the opposing wall of the penial sac as the animal matures (Fig. 177). The type I gland cells are far less numerous. They do not form a discrete gland and isolated cells are distributed regularly throughout the sarcobellum.

Viral infection of the sarcobellum

In one post-reproductive animal approximately 1 in 6 of the epithelial cells of the sarcobellum were infected with a virus. This virus is first seen in the nucleus (Fig. 232) and as it multiplies the nuclear membrane breaks down (Fig. 233) releasing large numbers of viral particles into the cytoplasm (Fig. 234).

An homogeneous material accumulates at the focus of multiplication (Figs. 232 & 233). It is immediately surrounded by pale, immature particles (diam. 0.15μm), but as the virus matures it migrates away from this central mass and develops an electron-dense core (diam. 0.1μm) (Figs. 235 & 236). The homogeneous material appears darker with age and by the time it is released from the nucleus irregular, electron-dense patches have developed (Fig. 237).

The dense-cored particles migrate towards the apex of the cell and the apical membrane begins to bulge outwards (Fig. 232). The virus is released in blebs of cytoplasm which are pinched off from the cell apex (Fig. 238), and with the disintegration of the nucleus cell death follows rapidly.

2. The penial sac

The epithelium of the penial sac is continuous with that of the sarcobellum. The ridges tend to be lower and more widely-spaced (Fig. 239), but ultrastructurally the epithelial cells are indentical (Fig. 240). Similarly, the connective tissue layer surrounding the penial sac is a continuation of the core of the sarcobellum and is composed of large numbers of muscle, gland and connective tissue cells in a dense collagen matrix (Fig. 241). Type II gland cells differentiate around the penial sac (Fig. 177), mirroring the distribution of these cells in the sarcobellum, but no type I cells are present here. Small bundles of 5 to 10 axons are scattered throughout the connective tissue layer while 1 or 2 larger nerves are seen at the surface. The penial sac is well vascularized and many small blood vessels are found at the perimeter of the connective tissue sheath.

3. The trifid appendage

As the penial sac tapers into the trifid appendage the epithelium loses its characteristic ridges to become quite smooth (Fig. 242) and the columnar epithelial cells are gradually replaced by those of the trifid diverticulum (Fig. 243).

In young animals (stage C) the trifid epithelial cells are basically cuboidal but the high level of infolding or pleating of the cell membranes results in the cells appearing somewhat irregular (Fig. 244). The large, basal nuclei also vary in shape (Fig. 245), each containing an eccentric nucleolus and scattered clumps of chromatin. The nucleus is surrounded by small, oval mitochondria and occasional strands of rough ER, while immediately above, lie several large Golgi bodies consisting of 7 to 10 concave cisternae (Fig. 246). A dense-staining secretory product accumulates within the innermost cisternae and these bud off small vesicles with an average diameter of 0.05μm. A few large, dense granules (max. diam. 0.3μm) accumulate sub-apically while small clear vesicles (diam. 0.05μm) are seen close to the cell surface.

The apical membrane bears numerous stubby microvilli and belt desmosomes (0.15μm thick) surround the cell apices (Fig. 247). At this stage of development the trifid diverticulum is flattened and the apices of the opposing cell ~~walls~~ membranes are compressed together.

During the D-E stages of development the trifid appendage expands and develops a distinct lumen (Fig. 248). The Golgi apparatus becomes very extensive and there is continuous production of small dense vesicles (diam. 0.05μm) (Fig. 249). The number of free vesicles in the

cell however does not appear to increase but the cell apices begin to swell and a pale homogeneous secretion accumulates apically (Fig. 250). The microvilli become stretched and, eventually, the apical membrane is completely smooth (Fig. 251). In many of the side branches the lumen becomes occluded by these swollen apices (Fig. 252) and where the apical membranes are in contact the mass of secretion coalesces. It is not clear if this swelling is an artifact, but techniques for both light and electron microscopy produce similar results.

The connective tissue sheath surrounding the trifid appendage is packed with circular or obliquely orientated muscle fibres, interspersed with occasional blocks of longitudinal muscle (Fig. 253). These muscle cells are closely applied to the bases of the epithelial cells and they follow the contours of the highly folded basal membranes. Pore cells and small nerves are scattered amongst the muscle fibres.

4. The vas deferens

The vas deferens is a slender duct which tends to become flattened against the wall of the oviduct (Fig. 254). The columnar epithelial cells have large basal nuclei, each containing a prominant nucleolus and scattered chromatin (Fig. 255). Strands of rough ER and a few

small mitochondria surround the nucleus while immediately above, lies the Golgi apparatus. The Golgi stacks are slightly concave and contain 7 to 8 slender cisternae (Fig. 256), their rims surrounded by small, pale-staining vesicles (diam. 0.05μm). Larger vesicles (max. diam. 0.1μm) are also present, and some appear to be associated with the outer Golgi elements. Clear vesicles (diam. 0.05 - 0.1μm) and electron-dense granules are seen in the sub-apical zone, and short chains of vesicles (diam. 0.05μm) lead to or from the cell surface (Fig. 257). In older animals (Stages F-H) lysosomes are common in the region of the Golgi apparatus (Fig. 255).

The apical membrane bears both microvilli and cilia and many cylindrical mitochondria are concentrated in the vicinity of the long ciliary rootlets (length approx. 2μm). Belt desmosomes (0.2μm thick) surround the apex of each cell and septate desmosomes extend for a further 1.0μm (Fig. 257). Hemidesmosomes are present at regular intervals along the basal membranes.

The connective tissue sheath surrounding the vas deferens is highly muscular and the obliquely orientated fibres are closely packed together (Fig. 258). Occasionally, neurons and small bundles of axons are seen within the muscle layer but the majority lie at the periphery where they form a superficial nerve plexus. In addition, a single large nerve (diam. 20 - 30μm)

runs along the whole length of the vas deferens. Pigment cells are scattered throughout the connective tissue sheath frequently lying alongside the nervous tissue, pore cells are rarely seen.

5. The oviduct

The epithelium of the oviduct has alternating patches of tall, columnar and low, cuboidal cells (Fig. 259) but apart from this obvious variation in shape the epithelial cells have similar ultrastructure.

The large, irregularly shaped nuclei each contain an eccentric nucleolus plus similar sized clumps of chromatin (Fig. 260). A well developed Golgi complex lies immediately above the nucleus and the curved stacks of 4 or 5 cisternae are surrounded by pale-staining secretory vesicles, 0.05 to 0.5μm in diameter (Fig. 261). These vesicles accumulate at the apex of the cell and their contents appears to be released into the duct lumen by exocytosis. Small pinocytotic vesicles (diam. 0.05μm) and MVBs (av. diam. 0.5μm) are also present near the cell surface (Fig. 260). Numerous small mitochondria are concentrated in the sub-apical region, close to the Golgi apparatus. The rough ER, however, is insignificant and few cisternae are seen around the nucleus.

The apical membrane has a border of microvilli, and belt desmosomes (0.2μm thick) with adjacent zonulae adhaerentes (1.5μm thick), surround the apex of each cell. Hemidesmosomes are present along the basal membranes. (Fig. 260).

As the animal matures secretory cells differentiate from unspecialized connective tissue cells. The nucleus, in common with other secretory cells associated with the reproductive tract, contains numerous small clumps of chromatin which give it the characteristic spotted appearance (Fig. 262). It is surrounded by short, slender strands of rough ER and many small, cup-shaped Golgi bodies with only 4 to 5 cisternae. A large secretory vesicle lies at the centre of each Golgi stack, and once these are released into the cytoplasm they coalesce to form a single secretory mass (Fig. 262). As this accumulates the secretory cells grow towards the duct lumen (Fig. 263), compressing the epithelial cells so that their apices bulge outwards and the whole epithelium appears very ragged (Fig. 264). The nucleus becomes flattened against the basal membrane and the cytoplasm is restricted to a narrow band around the cell periphery. The gland cells are now bulbous and the large amount of secretion makes the tissue very unstable in the electron microscope.

The highly muscular connective tissue sheath is dominated by the large mass of sub-epithelial secretory cells. A thin layer of longitudinal muscle closely follows the contours of the epithelium (Fig. 259) but beyond this the muscle fibres are randomly orientated, filling the interstitial spaces between the gland cells (Fig. 263). At the periphery, however, there is a broad band of circular muscle, which completely ensheaths the oviduct, and blocks of longitudinally orientated fibres are sparsely distributed around the outer edge (Fig. 265) Pigment cells are dispersed throughout these muscles layers.

The oviduct is not highly innervated but small nerves and, occasionally, large peripheral neurons are scattered throughout the connective tissue. Those nerves lying close to the epithelium are frequently filled with dense-cored granules (Fig. 266).

6. The bursa copulatrix

At the proximal end of the bursa copulatrix the epithelium is highly folded (Fig. 267) and the columnar epithelial cells appear very tall and thin, many with slightly bulging apices (Fig. 268). The nucleus lies towards the base of the cell and contains a central nucleolus with irregular clumps of chromatin. The rough ER synthesizes a pale-staining, fine-grained material

(Fig. 269). As this accumulates, the cisternae become very swollen, and large secretory granules bud from smooth, ribosome-free cisternae. The Golgi apparatus is also highly developed and cup-shaped Golgi bodies, each with around six cisternae, produce small, electron-opaque vesicles (diam. 0.05 - 0.1μm). These appear to fuse with the secretory granules arising from the ER and large, pale-staining vesicles accumulate at the apex of the cell. Omega bodies in the apical membrane and small, clear vesicles (diam. 0.05μm) at the cell apex, suggest pinocytotic activity (Fig. 270). Multivesicular bodies and small electron-dense granules (diam. 0.15 - 0.3μm) are also present.

As the secretion accumulates the cell apices swell outwards (Fig. 270). The apical membrane bears numerous microvilli and as it stretches those microvilli at the periphery become more widely spaced while those in the centre appear thicker and may develop complex configurations (Fig. 271).

The cytoplasm contains large numbers of polysomes, and many small, cylindrical mitochondria surround the nucleus and Golgi apparatus. Belt desmosomes, approximately 0.2μm thick, surround the apex of each cell and a zonula adhaerens extends for a further 1μm. Hemidesmosomes are commonly seen along the basal membranes.

As the lumen becomes filled with cellular and non-cellular debris the bursa expands and the folds of the epithelium diminish (Fig. 272). At the distal end of the saccule the epithelial cells are ciliated (Fig. 273). The ciliated and unciliated cells are similar, although the former appear to synthesize fewer vacuoles, and blebs in the apical membrane are rarely seen (Fig. 274). Characteristically, numerous mitochondria are present around the long ciliary rootlets.

The connective tissue sheath surrounding the bursa is very muscular and blocks of longitudinally orientated fibres alternate with bands of circular or oblique muscle (Fig. 275). Small groups of 5 or 6 neurons are seen around the periphery but the nervous tissue is not extensive and the axon bundles are small, rarely penetrating the thick muscle layer. Large pore cells and elongated pigment cells are scattered throughout the connective tissue sheath. The pigment cells are frequently found alongside the nerves and neurons at the periphery.

ULTRASTRUCTURE OF THE ANTERIOR GENITAL DUCTS IN A.HORTENSIS

1. The lower atrium

The "spongy" gland surrounding the lower atrium synthesizes lipid and to ensure adequate penetration of fixative and embedding medium tissue prepared for electron microscopy could not exceed 100μm in any plane. This made it impossible to study the epithelium of the lower atrium and the following description is limited to the ultrastructure of the secretory cells.

The secretory cells differentiate from mesenchymal cells in the sub-epithelial connective tissue. Their large prominent nuclei each contain a central nucleolus and chromatin lines the nuclear envelope. The cytoplasm appears granular due to the high concentration of polysomes and an heterogeneous assortment of clear vesicles (diam. <0.1μm). Isolated rough ER cisternae and small, oval mitochondria surround the nucleus, but Golgi bodies cannot be recognized at this stage.

The start of secretory activity is indicated by the formation of clear-staining plates or rods (Fig. 276.) These are associated with smooth, tubular ER, common to lipid secreting cells. This smooth ER

proliferates, eventually filling the cytoplasm (Fig. 277), and as synthesis proceeds numerous small Golgi bodies can be identified, their 5 to 6 cisternae swollen with clear secretion (Fig. 278). Clear vesicles and developing, plate-like secretory granules are released into a central secretory mass. The electron-opaque granules now appear crystalline and are partially bisected. They are suspended in a clear matrix (Fig. 279).

As the secretion accumulates, the cytoplasm becomes restricted to a narrow band around the periphery of the cell (Fig. 279). The nucleus now lies basally, and the apices grow towards the epithelium (Fig. 280) where they push between the epithelial cells and eventually release their contents into the atrium.

2. The vas deferens

The slender vas deferens is a simple region of the male duct lined by columnar epithelial cells (Fig. 198). The basal nuclei are lobed and each contains a central nucleolus plus large, scattered clumps of chromatin. In young animals granular accumulations, probably glycogen, are seen immediately above and below the nucleus (Fig. 281), but they disappear with age.

The rough ER becomes filled with a pale-staining secretion and the cisternae nearest the Golgi apparatus often appear swollen or vesicular. Several, concave Golgi bodies lie above the nucleus, each consisting of 4 to 6 cisternae (Fig. 282). The outer cisternae are swollen and filled with a pale material while the inner cisterna is normally slender, with electron-opaque contents. Small, electron-opaque vesicles (av. diam. 0.05μm) accumulate at the inner surface, and repeated fusion with larger pale vacuoles, possibly arising directly from the ER, results in the formation of pale-staining secretory granules (max. diam. 1μm). As these granules condense they become electron dense with an average diameter of 0.5μm. They accumulate at the apices of the cells (Fig. 283).

Small, clear vesicles (av. diam. 0.05μm) are present at the cell apex and MVBs are occasionally seen. The apical membrane bears microvilli interspersed with long cilia. The ciliary rootlets extend into the cytoplasm for approximately 2μm and are surrounded, characteristically, by many cylindrical mitochondria. Belt desmosomes (0.5μm thick) surround the apex of each cell and septate desmosomes are present at frequent intervals between the longitudinal membranes. Numerous small hemidesmosomes are seen at the cell bases.

The cytoplasm is rich is polysomes and in some cells lipid droplets are seen around the nucleus.

The connective tissue surrounding the vas deferens differs from that of D.reticulatum and lacks its thick muscular sheath (Fig. 284). The muscle cells are more dispersed, and their fibres are randomly orientated. Neurons and small nerves form a superficial nerve plexus and pore cells are seen around the periphery. Large cells containing either lipid or electron-dense granules are also present.

One animal was infected with spirochaetes and the helical-shaped bacteria were seen in clusters throughout the connective tissue matrix (Fig. 285).

3. The epiphallus

The inner surface of the epiphallus is thrown into numerous folds, forming rounded, knob-like papillae (Figs. 286 & 287). The columnar epithelial cells are highly interdigitating and the central nuclei are irregularly lobed (Fig. 288). Each nucleus contains a central nucleolus and heterochromatin lines the nuclear envelope. The cytoplasm is displaced by large accumulations of granules (av. diam. 0.05μm) of varying density, which give the cells a mottled appearance (Figs. 288 & 289). The organelles are generally

restricted to the periphery of the cell and there is no indication of active synthesis. Large, apparently empty vacuoles are scattered throughout the cells (Fig. 289) and, occasionally, secondary lysosomes are seen. Small vesicles (diam 0.05μm) are present at the cell apex and omega bodies may be observed in the apical membrane.

The anterior portion of the epiphallus is densely fringed with microvilli (Fig. 289). Towards the vas deferens, however, the epithelial cells suddenly become ciliated (Figs. 286 & 287) and large mitochondria are concentrated at the apices. In all other respects the ciliated and unciliated cells are similar. Belt desmosomes (0.25μm thick) are present around the apex of each cell and zonulae adhaerentes extend for a further 3μm. Hemidesmosomes are frequently seen at the cell bases. Both the longitudinal and basal membranes show complicated infolding.

Calcium cells lie immediately beneath the epithelium (Figs. 288 & 290) and fill the centre of each papilla (Fig. 287). These large cells have irregularly-shaped, chromatin rich, central nuclei but all other cytoplasmic detail is lost due to the presence of calcium concretions (Fig.291).

The connective tissue sheath surrounding the epiphallus is highly muscular (Fig. 290) with layers of circular muscle interspersed with occasional pigment cells and granular connective tissue cells, similar to those seen around the vas deferens. Large nerves and occasional neurons are present at the periphery.

4. The oviduct

In addition to the obvious difference in musculature, the thick and thin sections of the oviduct differ from one another ultrastructurally.

Thick oviduct

The thick oviduct is lined by tall, columnar epithelial cells with large, irregularly shaped basal nuclei (Fig. 292). The nuclei contain rounded clumps of chromatin, similar in size to the nucleolus, and are surrounded by the slender rough ER. Concave or cup-shaped Golgi bodies lie immediately above the nucleus, each consisting of between five and eight cisternae (Fig. 293). Large, pale vesicles form at the inner surface, possibly by distension of the innermost cisterna. The vesicles accumulate sub-apically and may fuse with one another to form larger secretory vacuoles.

Only a few secretory vesicles extend into the apex of the cell (Fig. 294). Here the cytoplasm contains numerous small, clear vesicles (av. diam. 0.05μm) plus one or two MVBs. Small cylindrical mitochondria tend to be concentrated around the Golgi apparatus, and the cytoplasm appears granular due to the large number of polysomes.

The cells are unciliated but the apical membrane bears long microvilli, up to 2μm in length (Figs. 292 & 294). Belt desmosomes (0.2μm thick) surround the apices of the cells and zonulae adhaerentes extend for a further 1.5μm. The basal membranes are very folded, tapering into thin processes (Fig. 295) which are attached to the basement membrane by small hemidesmosomes.

A well-developed nerve plexus lies beneath the epithelium and many large neurons penetrate between the epithelial cells (Figs. 292 & 295), their axons running along grooves in the basal membranes.

The connective tissue immediately surrounding the oviduct is loosely packed with longitudinally orientated muscle, nerve and occasional pigment cells (Fig. 292). Further out, however, the density of the muscle increases and circular muscle fibres are present (Figs. 296 & 297). The bands of circular muscle thicken at the

expense of the longitudinal muscle, which now becomes organized into single cell layers (Fig. 298). Towards the periphery, however, the longitudinally orientated muscle increases and eventually surrounds the thick muscle sheath (Fig. 296). The muscle cells establish firm attachments with one another and with the underlying connective tissue, and both desmosomes and hemidesmosomes are frequently seen on the sarcolemma (Fig. 299).

No neurons are found between the muscle layers but they are present around the outer surface where they form a peripheral nerve plexus (Fig. 300). This connects to the inner plexus by a network of nerves which run between the muscle cells and establish many neuromuscular junctions (Fig. 299).

Pigment cells are scattered throughout the muscular sheath (Fig. 298).

Thin oviduct

In contrast, the thin oviduct is ciliated and the secretory vesicles are absent (Fig. 301).

The columnar epithelial cells have large, rounded, basal nuclei, rich in heterochromatin. Each nucleus is surrounded by long cylindrical mitochondria and slender

rER cisternae. Above this lies a well developed Golgi apparatus, each concave stack consisting of 5 to 6 cisternae, and small, clear vesicles (av. diam. 0.05µm) accumulate at the inner face and in the neighbouring cytoplasm (Fig. 302).

Mitochondria are concentrated between the Golgi zone and the cell apex. Small granular accumulations, possibly glycogen, are present in this sub-apical region, together with round, lipid-like droplets and MVBs. Clear vesicles (diam. 0.05 - 0.15µm) are seen near the cell apex. The cilia have long, striated rootlets which extend approximately 2.5µm into the cell, but the microvilli are shorter than those of the thick oviduct and do not exceed 1µm in length. Belt desmosomes (0.4µm thick) with underlying zonulae adhaerentes (1.3µm long) surround the apices of the cells. The basal membranes show little infolding and hemidesmosomes are present at regular intervals along their length.

The connective tissue surrounding the thin oviduct is loosely packed with muscle, nerve and occasional pigment cells (Fig. 303), and resembles the inner layer of the thick muscular sheath described above. The muscle fibres are either longitudinally or obliquely orientated and circular muscle is absent. Large neurons with their associated nerves form a diffuse nerve

plexus, but unlike those of the thick oviduct they do not establish intimate contact with the epithelial cells.

5. The bursa copulatrix

The contents of the bursa copulatrix created problems when preparing the tissue. Total removal of the digestive contents necessitates much handling and results in cell distortion, while material taken from young animals gives no indication of cell activity. Consequently the ultrastructure of the bursa is still unknown.

ANALYSIS OF THE LIPID IN THE LOWER ATRIUM OF A.HORTENSIS

The gland surrounding the lower atrium of A.hortensis develops during the C-D stages and in reproductively active animals it forms a distinct, white, sponge-like collar. The secretion gives a highly positive reaction with Sudan black B, indicating the presence of lipids, and this was confirmed biochemically using thin layer chromatography. The results are shown on the chromatogram trace (Fig. 304).

The ether extracts, B and C, are identical. Both contain a mixture of hydrocarbon, cholesterol ester, two methyl ester components based on different fatty acids,

triglyceride, two components of either tri- or di-glycerides, four separate regions of free fatty acids, a large and very dense sterol region, and finally phospholipid, which remains at the origin. A large U.V.-staining spot, associated with the triglyceride component, does not stain with iodine and is possibly a pigment of triglyceride.

The aqueous extract, A, shows a reduced range of lipid components, and lacks both hydrocarbon and cholesterol ester.

DISCUSSION

The arrangement of the pulmonate reproductive tract from the carrefour to the genital opening is the subject of much discussion. The degree of fusion and the presence of either two (diaulic) or three (triaulic) functional grooves or ducts has been postulated as an indication of phylogenetic development, based on the premise that the ancestral pulmonates possessed a single pallial duct incorporating a seminal groove for incoming sperm, a sperm groove for outgoing sperm and a female channel for transporting the ova (Visser, 1973). Examples of triauly are seen in some streptaxid snails e.g. Discartemon (Berry, 1965), while in D.reticulatum and A. hortensis the complete absence of a seminal duct is thought to represent the most advanced arrangement.

However, despite the different levels of fusion and the presence or absence of a seminal duct, the relationship of the anterior genital ducts shows little variation throughout the Stylommatophora (Fig. 305). The vas deferens opens into an expanded penis, penial mass or epiphallus before entering the genital atrium. The inner surface of this expanded region is highly elaborated with pleats, papillae etc., and a diverticulum may be present at the junction with the vas deferens. The oviduct opens directly into the genital atrium, with the bursa copulatrix arising near this junction.

Exceptions to this generalized plan are seen in aphallic individuals where the male copulatory organs are completely absent e.g. Acanthinula (Boycott, 1917), Vallonia (Whitney, 1940) and Deroceras laeve (Quick, 1960; Nicklas and Hoffmann, 1981). This condition is usually genetically (Watson, 1923) or environmentally (Nicklas and Hoffmann, 1981) controlled, although in the giant slug, Ariolimax, aphallism frequently results from mating, one partner biting off the everted penis of the other (Mead, 1943).

Differences in the morphological detail of the anterior genital ducts characterize the Stylommatophora and are necessary for species recognition at copulation. It is impossible to survey the complete range of form

and variety of appendages and glands that are associated with the copulatory apparatus, and this discussion will be limited to a comparison of limacid and arionid morphology, with comments on the closely related Testacellidae.

The copulatory organs of the Limacidae and Arionidae differ in two major respects. Firstly, the arionid slugs have no eversible penis and secondly, they lack a stimulator. A true penis, capable of eversion at copulation, is found in all limacid and testacellid species. In Milax this is preceded by a short epiphallus (Fig. 305f) while in Deroceras it is more usually known as the penial mass (Fig. 305h). The penial mass encloses a conical sarcobellum which is protruded during courtship and caresses the partner, stimulating it to respond. In Milax the stimulator is found in the genital atrium and in Limax, although there is no clearly defined protrusible structure, the everted penis, bearing longitudinal combs, functions similarly.

It has been suggested that the ligula of Arion are, in some way, comparable to the stimulators seen in the Limacidae (Quick, 1960). The ligula are associated with the oviduct, either as a thickened pad at the junction with the upper atrium, e.g. A. ater, or as parallel longitudinal folds in the anterior region of the duct, e.g. A. lusitanicus, A.subfuscus. They are

stiffened by a "cartilaginous" (sic) core (Quick, 1960). During courtship, however, these structures are not exposed and are only seen on the surface of the everted atria surrounding the entrance to the oviduct, in animals fixed at the moment of sperm exchange (Quick, 1960). This suggests that their function is one of attachment rather than stimulation.

Quick (1960) described the ligule of A. hortensis as paired longitudinal ridges in the thick oviduct, but these were not observed in any of the animals dissected for this study and the firm "cartilaginous" core was not apparent. The highly muscular nature of this section of the duct may have given a false impression of folds, or this may be a regional difference, since Quick (1960) comments on the variation in size and thickness of the ligule in British and Dutch slugs.

The term sarcobellum actually means dart sac, and from his work on the Urocyclidae Mol (1970) traces its development from a diverticulum in the lumen of the penis, capable of secreting a calcareous dart, to a separate glandular structure in the genital atrium. This indicates a possible relationship between the well-developed stimulators of Deroceras and Milax species and the dart sac described in Philomycus carolinianus (Kugler, 1965).

The trifid appendage is found only in Deroceras and its position at the junction of the vas deferens and penial mass suggests that it is comparable to the flagellum seen in just one member of the Testacellidae, Testacella haliotidea (Fig. 305d), but common throughout the Helicidae (Fig. 305b) and Bulimulidae (Fig. 305c). The function of this branched diverticulum will be discussed later, in relation to courtship and copulation.

Despite the wealth of information on the morphology of copulatory organs in the Stylommatophora, the ultrastructure is poorly documented, and cell detail is largely based on histological and histochemical observations (e.g. Noyce, 1973; Sirgel, 1973; Visser, 1973; Els, 1974; Stears, 1974).

The lower atrium

The sub-epithelial gland surrounding the lower atrium of A.hortensis appears empty after histological preparation and this vesicular appearance, together with its soft, pulpy texture, has given it the name "spongy gland".

A similar gland is present in A.ater . Histochemical staining with Sudan black B and Schultz's test has demonstrated the presence of lipids and sterols

(Lüsis, 1961; Smith, 1965) and Lüsis reports that lipid is first detectable in C-D stage animals.

Ultrastructurally, the secretory cells are typical of lipid-secreting tissues, e.g. mammalian liver, having a closely packed network of smooth, tubular ER. It is postulated that in the liver triglycerides are formed from fatty acids in the smooth reticulum and here they are combined with proteins, synthesized in rER, to form lipoprotein particles (diam. 0.03-0.1 μm). There is also evidence that the smooth ER plays an important role in the synthesis of cholesterol.

In the spongy gland, however, the rER is insignificant and the secretion combines with that of the Golgi apparatus forming irregular, ellipsoid crystals, suspended in an homogeneous matrix. These crystals, with a maximum diameter of 2.5 μm, are considerably larger than the lipoprotein particles seen in the liver.

Thin layer chromatography on the crude extract of spongy glands, taken from adult A.hortensis, indicates that this secretion contains a complicated mixture of lipids. This confirms an earlier analysis of the spongy gland in A.ater (Bayne, 1966), although these extracts contained no fatty esters which comprise approximately one third of the total lipid in A.hortensis. This

difference is probably due to variations in the initial extraction and/or in the solvent used for chromatography. Further analysis of this secretion is necessary to determine the biochemical nature of the crystalline and matrix components.

The function of this lipid is not clear. Smith (1965) postulated that it may act as a lubricant during egg-laying and possibly copulation, but Bayne's findings that a similar lipid coats the eggs of A.ater, suggest that its major contribution is to the egg-shell layers (Bayne, 1966).

Histologically, the secretory cells of this spongy gland closely resemble those surrounding the free oviduct in D.reticulatum, and the question arose whether these two glands were homologous. The oviducal gland, however, does not stain with Sudan black, and lipid cannot be extracted in diethyl ether. Therefore it can be concluded that there is no comparable lipid-secreting gland in D.reticulatum.

The vas deferens

Throughout the Pulmonata the vas deferens is described as a simple, ciliated duct surrounded by layers of muscle. This description also applies to D.reticulatum and A.hortensis, and superficially these

ducts appear similar and completely unspecialized. At the ultrastructural level, however, the epithelial cells of A.hortensis appear very active with enormous accumulations of dense granules filling their apices. The pale contents of the outer Golgi cisternae suggests that secretion produced by the ER is further processed within the Golgi stack, giving rise to the dense vesicles around the inner face. Alternatively, or perhaps in addition, some of the larger, pale vesicles may arise directly from the ER. Exocytosis of these secretory granules was not observed, although the presence of clear, probably pinocytotic, vesicles and MVBs suggests recent activity at the cell surface. Possibly, release of the granules is stimulated during courtship where they may contribute to the formation of the spermatophore.

In D.reticulatum no such secretion is observed. The cells show high levels of pinocytotic activity, with the formation of secondary lysosomes, suggesting that many of the small vesicles produced by the Golgi apparatus are lysosomal. Continuous pinocytosis has been observed before in the reproductive tract, e.g. hermaphrodite duct and talon, where it has been suggested that it removes excess fluid in the duct lumen. Similar control may be necessary here, particularly following preparation of the sperm package. Jong-Brink (1969) reports similar pinocytotic activity in the epithelial

cells lining the vas deferens and penial complex of Biomphalaria glabrata and suggests that this increases cell turgor which assists in everting the copulatory organs.

The thick, muscular sheath which surrounds the vas deferens of D.reticulatum is considerably reduced in A.hortensis where the muscle fibres are dispersed and randomly orientated. This difference may reflect greater distension and/or elongation of the duct in D.reticulatum at copulation.

The penial mass and epiphallus

Throughout the Stylommatophora the sperm packages are prepared in the penis sac or epiphallus, the linings of which are highly elaborate (c.f. the Australian camaenid snails, Solem, 1979) and mould the spermatophore, giving it the characteristic surface architecture.

In A.hortensis the papillated epithelium is ciliated posteriorly, while the anterior half bears only a fringe of microvilli. The cilia may serve several functions: (1) circulation of the secretion, (2) propulsion of the sperm, secretion and possibly the completed spermatophore anteriorly, and (3) they may prevent the tail of the spermatophore adhering to the

epiphallus. In D.reticulatum only the cells at the base of the ridged epithelium are unciliated. Type I secretory cells release their secretion into these grooves on the surface of the sarcobellum, but there is no obvious reason to account for the absence of cilia.

In both species there is evidence of high levels of pinocytosis, with omega bodies and vesicle chains frequently seen in and around the apical membrane. Intercellular vacuoles in the epithelium of D.reticulatum suggest that there is some accumulation of fluid, possibly as a direct result of continuous pinocytosis. Alternatively this may indicate that fluid is being pumped across the epithelium either to equilibrate internal/external pressures or to raise the turgidity of the connective tissue and thus aid eversion of the penis sac (Hubendick, 1948). In A.hortensis the large intracellular vacuoles do not appear to be involved in fluid movement.

The epithelial cells are not secretory and in common with other cells displaying high levels of pinocytosis, primary lysosomes presumably arise from the Golgi apparatus.

In A.hortensis the granular accumulations are probably glycogen. This would agree with the histochemical observations on the epiphallus of A.ater

where glycogen was located using P.A.S., and treatment with diastase (Smith, 1965). Similarly, in Philomycus carolinianus, Kugler (1965) has demonstrated that glycogen is present throughout the epithelium of the reproductive tract.

The papillae of the epiphallus are packed with calcium cells. Large, rounded, empty-looking cells have also been observed in the loose, reticular connective tissue surrounding the epiphalli of some helicid snails (Matthes, 1915; Baecker, 1932; Noyce, 1973) and the slugs, Milax gagates (Els, 1974) and Arion ater (Smith, 1965), and their appearance under the light microscope led workers to assume that they were secretory. Considering the present findings it would be reasonable to assume that these cells also contain stores of calcium, but histochemical tests on the epiphallus of A.ater gave no calcium reaction, and it remains to be seen if this is a common site for calcium storage.

Both Cepaea and Helix contain calcium crystals in the cuticle of the penis, but large calcium storage cells are more usually found associated with the female ducts e.g. A.ater (Smith, 1965) or blood vessels e.g. D.reticulatum (Duval, 1981), since calcium is mobilized in the blood during egg-laying (Tompa and Wilbur, 1977). The significance of calcium storage around the

epiphallus is not clear, but perhaps the cells give some structural support, maintaining the shape of the papillae when the duct fills with sperm, while at the same time providing a local supply of calcium for the egg-shell layers.

The epiphallus of A.hortensis is completely aglandular. In contrast, a large, mucus-secreting gland is present in the penial mass of D.reticulatum . This appears to release secretion continually which must act as a lubricant both at rest and during copulation.

The sarcobellum is a solid, muscular organ with a central blood sinus, and during copulation it is erected by increasing the blood pressure. The muscular core provides mobility, and as it plays over the partner during courtship secretion is probably released from both types of gland cell. While the major role of these secretions is one of lubrication, type I and/or type II secretions may also contain some excitatory substance, stimulating both the partner and other slugs which may come into contact with it.

Both epiphallus and penial mass are surrounded by a muscle sheath. This is particularly well developed around the epiphallus where muscular contraction may be necessary for ejecting the spermatophore. The penial mass on the other hand has no ejaculatory role.

However, it does have a great capacity to stretch, the everted sac showing a fivefold increase in size, and adaptations for considerable expansion are seen in the numerous junctional complexes which are necessary for intercellular adhesion, as well as attaching the epithelium to the underlying connective tissue.

The ultrastructure of the trifid appendage does not clarify its function. The accumulation of fluid, causing distortion of the cell apices, is normally indicative of apocrine secretion. This was never observed, however, and it can only be assumed that the secretion is released during courtship or copulation.

The oviduct

The term oviduct is usually applied to the female duct extending from the common duct to the junction with the bursa copulatrix. Anteriorly, it becomes the vagina, but in D.reticulatum and A.hortensis, where the bursa duct opens directly into the genital atrium, no true vagina exists. This morphological definition simplifies the nomenclature of the anterior female ducts but some workers have based their terminology on comparative histology (Noyce, 1973; Sirgel, 1973; Els, 1974) with the result that oviduct and vagina have become confused.

In the slugs, Philomycus carolinianus (Kugler, 1965), Limax valentianus (Stears, 1974) and Milax gagates (Els, 1974) the oviduct is composed of two histologically different regions. Posteriorly it is simply a continuation of the oviducal groove of the common duct, but anteriorly the oviducal gland cells diminish and the surrounding connective tissue becomes very muscular. In P.carolinianus and M.gagates development of the muscular sheath is accompanied by loss of cilia. This shows certain similarities with the oviduct of A.hortensis, but here the ciliated, posterior oviduct is thin and completely aglandular while anteriorly the unciliated, thick oviduct is lined by secretory epithelial cells.

The unciliated, glandular ducts of D.caruanae (Sirgel, 1973) and Theba pisana (Noyce, 1973) have both been termed the vagina, based on their histological similarity to the vagina of Oxychilus cellarius (Rigby, 1963). In these animals the oviduct is supposedly absent, although Sirgel (1973) suggests that in D.caruanae it is incorporated into the common duct, forming the anterior portion of the oviducal groove. For the reasons given above, the same glandular, unciliated region in D.reticulatum is known as the oviduct. These ducts, however, appear to be homologous, having similar, pear-shaped, sub-epithelial gland cells and a secretory epithelium.

Thus, histological comparison of the oviducts reveals certain, repeated characteristics. Firstly, the duct is highly muscular, although the layers of muscle may be interrupted by sub-epithelial gland cells, and secondly the epithelium loses its cilia as it continues anteriorly, and becomes lined by unciliated, secretory epithelial cells.

The large secretory cells associated with the oviduct of D.reticulatum are histochemically distinct from the oviducal gland cells in the common duct and therefore this region must be considered as a separate, carbohydrate-secreting gland. It closely resembles the capsule gland of Ariophanta ligulata which Dasen (1933) suggests secretes the hard, outer, egg-shell layer, and a similar function can probably be attributed to the gland of Deroceras species.

The accumulation of vesicles in the secretory epithelial cells indicates that the rate of their formation far exceeds the rate of exocytosis. In the thick oviduct of A.hortensis the main store of secretion lies sub-apically and only one or two isolated vesicles are present near the apical membrane. This favours the concept of simultaneous exocytosis, possibly regulated by ovipository activity.

In D.reticulatum, however, the vesicles usually lie at the cell apex and in many animals there is evidence for continuous, slow release. The function of this constant, low-level secretion is not obvious, and possibly the rate of exocytosis increases during egg laying, resulting in a massive discharge which may contribute to the eggs or facilitate their passage through the duct.

Since continous pinocytosis appears to be a common feature throughout the reproductive tract, the vesicles seen in the apices of the secretory cells probably arise from the steady uptake of fluid, as well as the direct result of exocytotic activity.

In M.gagates (Els, 1974) and L.valentianus (Stears, 1974) localized thickening of the muscular sheath, near the junction with the genital atrium, has been likened to the sphincter described in Oxychilus cellarius (Rigby, 1963). While Sirgel (1973) suggests that in D.caruanae circular muscle surrounding the anterior region of the common duct functions similarly, preventing the descending sperm and prostate secretion entering the "vagina", although he does not consider how this can seal the female groove and still leave the male groove open. There is no evidence to indicate that sphincters are present in either D.reticulatum or A.hortensis.

The well-developed musculature may be essential for expelling the newly formed eggs into the genital atrium.

The bursa copulatrix

The bursa copulatrix is histologically similar throughout the pulmonates. It is a sac-like organ, lined by tall, columnar epithelial cells whose activity appears to be stimulated by receipt of material. Horstmann (1955) demonstrated that in Lymnaea stagnalis the presence of the sperm mass stimulates the epithelial cells to release a proteinase, which progressively breaks down the sperm, resulting in an increase in the free amino acid content of the sac. Digestion continues for two days before the epithelium starts to resorb the soluble material. Similar observations on Helix pomatia indicated that the bursa copulatrix became swollen immediately after reproductive activity (Lind, 1973). Following copulation the spermatophore and surplus sperm were removed within one week, but the excess secretions of dart shooting and egg-laying were resorbed in only two days.

The epithelium lining the bursa copulatrix consists of ciliated and non-ciliated cells. The latter show high levels of secretory activity and secretory granules, produced by fusion of ER- and Golgi-derived

vesicles, accumulate at their apices, which become greatly distended. This is indicative of apocrine secretion. The cells contain high levels of ribonuclease, deoxyribonuclease and acid hydrolase (Németh and Kovács, 1972; Reeder and Rogers, 1979). Since secondary lysosomes do not accumulate in D.reticulatum it can be assumed that the secretory granules contain the enzymes for extracellular digestion, and pinocytotic activity may indicate resorption of the digested material.

In Milax gagates (Els, 1974), Limax valentianus (Stears, 1974) and Deroceras caruanae (Sirgel, 1973) the two cell types are generally interspersed, with ciliated cells concentrated at the apex. They are clearly segregated in D.reticulatum and A.hortensis, however, where the proximal half of the saccule is uniformly lined by secretory cells, and ciliated cells are only present distally. Stears (1974) suggests that the ciliated cells represent the inactive or resting state, and stimulation of secretory activity results in distension of the cell apices with simultaneous loss of cilia. Alternatively the ciliated cells may persist, forming bands along or around the saccule which circulate the bursa contents.

In both D.reticulatum and A.hortensis the bursa duct is not defined histologically and is only indicated by constriction of the saccule near the genital atrium, where the muscular connective tissue may appear slightly thicker. In other species, where the duct is elongated, e.g. Gonaxis gwandaensis (Visser, 1973) the epithelium is usually ciliated and shows no secretory activity.

A combination of ciliary movement and peristalsis conveys the waste products of reproduction to the digestive bursa (Horstmann, 1955; Lind, 1973).

The nerve plexus

A diffuse nerve plexus surrounds the anterior genital ducts of both D.reticulatum and A.hortensis, suggesting that there is some nervous regulation of reproductive activity. In A.hortensis this is particularly well-developed around the thick oviduct where neurons establish intimate contact with the epithelium. Possibly the presence of eggs in this region of the reproductive tract stimulates some other related behavioural, secretory or biochemical process.

DISCUSSION ON VIRUS-LIKE PARTICLES FOUND IN THE SARCOBELLUM OF D. RETICULATUM

Much of the information on invertebrate viruses is restricted to the class Insecta and very few references have been made to viruses which infect molluscs. This does not mean, of course, that insects are more susceptable to viral infection than other invertebrates, but just reflects the number of virologists working in the different fields.

Within the Mollusca reports are limited to studies on two cephalopod and one bivalve species. Devauchelle and Vago (1971) observed virus-like particles in the stomach epithelium of Sepia officinalis and their structure and pattern of development suggest that they are closely related to the Rheoviridae. Other virus-like particles have been found in Octopus vulgaris (Rungger et al., 1971). They are associated with oedematous tumours which develop within the muscles of some animals and have been likened to the Iridoviridae (Tinsley and Harrap, 1978). Yet another type of virus is seen in the tissues surrounding the haemolymph sinuses of the oyster, Crassostrea virginica (Farley et al., 1972). The viral particles are morphologically similar to herpetoviruses and large aggregates are found within the nuclei of infected cells. These particles are usually hexagonal (diam.

70 - 90nm) and while many contain an electron-dense core others appear empty. The electron micrographs indicate a close similarity between these particles and those found in D.reticulatum.

The size, appearance and site of multiplication of the virus-like particles present in the sarcobellum of D.reticulatum suggest that they too are related to the Herpetoviridae (Tables 1 & 2). However, they appear spherical in the electron microscope at magnifications of up to 60,000 x and there is no clear evidence for icosahedral symmetry. Apparently this is a common misinterpretation with preliminary studies (Hsuing, 1982) and more specific examinations will usually reveal the typical hexagonal cross-section. This may also be true for the slug virus.

These virus-like particles were only observed in the epithelial cells of the sarcobellum and the rest of the reproductive tract was unaffected. Unfortunately other tissues were not examined, so the extent of the infection in this one animal is unknown. The mode of infection is not clear, but the virus appears to penetrate the nucleus, where it replicates, resulting in large numbers of identical cored particles. The nuclear membrane breaks down, liberating these particles into the cytoplasm. They migrate apically and are released at the apical membrane in blebs of cytoplasmic material.

Table 1 Characteristics of the virus families

Family	Diameter (nm)	Symmetry of nucleocapsid	Nucleic Acid	Envelope	Site of Multiplication
ADENOVIRIDAE	70-90	Icosahedral	DNA	-	Nucleus
ARENAVIRIDAE	50-300	Helical	RNA	+	Cytoplasm
BACULOVIRIDAE	40-70x250-400	Bacciliform	DNA	+	?
BUNYAVIRIDAE	100	Filament	RNA	+	Cytoplasm
CORONAVIRIDAE	70-120	Helical	RNA	+	Cytoplasm
HERPETOVIRIDAE[1]	150-200	Icosahedral	DNA	+	Nucleus
IRIDOVIRIDAE	130-300	Icosahedral	DNA	+	Cytoplasm
ORTHOMYXOVIRIDAE[2]	100	Helical	RNA	+	Cytoplasm
PAPOVAVIRIDAE	45-55	Icosahedral	DNA	-	Nucleus
PARAMYXOVIRIDAE	150-300	Helical	RNA	+	Cytoplasm
PARVOVIRIDAE	18-26	Icosahedral	DNA	-	Nucleus
PICORNAVIRIDAE	20-30	Icosahedral	RNA	-	Cytoplasm
POXVIRIDAE	170-260x300-450	Icosahedral	DNA	+	Cytoplasm
REOVIRIDAE	60-80	Icosahedral	RNA	-	Cytoplasm
RETROVIRIDAE	100	Icosahedral	RNA	+	Nucleus
RHABDOVIRIDAE	130-300x70	Helical	RNA	+	Cytoplasm
RUBIVIRIDAE[3]	60	Icosahedral	RNA	+	Cytoplasm
TOGAVIRIDAE	40-70	Icosahedral	RNA	+	Cytoplasm

1. Also known as Herpesviridae
2. Also known as Myxoviridae
3. May be included with the Togaviridae

(Hsuing, 1982; Primrose and Dimmock, 1980; Roizman and Furlong, 1974)

Table 2

Comparison of the virus seen in D. reticulatum with the Herpetoviridae

	Herpetoviridae	Virus of D. reticulatum
Diameter of virion (nm)	150-200	150
Diameter of capsid (nm)	85-110	100
Envelope	Present	Present
Symmetry of nucleocapsid	Icosahedral	Icosahedral?
Site of multiplication	Nucleus	Nucleus

Presumably these infected blebs of cytoplasm remain in the mucus which bathes the evertile penial mass and transmission of the virus may occur during copulation.

In C.virginica the virus appears to stimulate cell proliferation while in O.vulgaris the infected tissue is recognised by the appearance of intramuscular tumours. In both species death follows a few months after infection. Although the infected cells of the sarcobellum became very enlarged there was no suggestion of increased cell multiplication or swelling of the surrounding tissues. Proliferation of the virus however destroyed the cell structure and this suggests that it is also a lethal infection.

It is generally thought that true members of the Herpetoviridae multiply solely within vertebrate species (Primrose and Dimmock, 1980). Viruses which are common to plants have not been found in animal tissues, and vice versa, but it seems an arbitrary preference for a virus to infect animals with backbones rather than those without. The majority of viruses have been identified in both vertebrate and invertebrate species (Tinsley and Harrap, 1978; Primrose and Dimmock, 1980) and those which appear specific to one or the other probably just reflect an incomplete knowledge of their full range of hosts. Since two cases of herpes-like viruses have now

been found within the Mollusca it seems that this is certainly true for the Herpetoviridae.

DISCUSSION ON MORPHOGENESIS OF THE STYLOMMATOPHORAN REPRODUCTIVE TRACT.

Since this investigation is not concerned with the origin of the reproductive tract, the unitarist versus dualist theories (see Laviolette, 1954; Martoja, 1964; Tardy, 1971; Luchtel, 1972) will not be discussed. However, in view of Visser's recent observations that the reproductive tract of Gonaxis gwandaensis originates post-embryonically (Visser, 1973) it is worth while noting that a reproductive tract, albeit a simple tube, is present in newly-hatched D.reticulatum and A.hortensis. This confirms its embryonic origin and agrees with other reports on Stylommatophora, namely, the helicid snails Helix pomatia and H.nemoralis (Jhering, 1975) and the slugs, Agriolimax (=Deroceras) agrestis (Brock, 1886), Limax maximus (Hoffmann, 1920) and Arion ater (Pabst, 1914; Laviolette, 1954; Lüsis, 1961).

The timing of morphogenesis is not usefully compared since the life-histories of different species vary considerably, but in the few reports relating reproductive tract and gonad development there is

considerable correlation with the observations on D.reticulatum and A.hortensis (c.f. Lüsis, 1961; Visser, 1973).

Expansion of the rudimentary albumen gland during the spermatocyte or B-stage of development precedes the formation of a talon, and this distension in the simple tube-like reproductive tract locates the carrefour in young animals. Subsequent growth and development of the albumen gland (Pabst, 1914; Hoffmann, 1920; Lüsis, 1961) and differentiation of the stylommatophoran talon (Hoffmann, 1920; Lüsis, 1961; Visser, 1973) closely follow the descriptions given for D.reticulatum and A.hortensis, and these two species provide a model for the development of diverticulate and looped carrefours respectively.

Division of the anterior region of the immature reproductive tract, into two separate, male and female ducts, also occurs during the B stage, thus coinciding with differentiation of the talon (Lüsis, 1961). The origin of the bursa copulatrix, appears to be somewhat variable. In those species where the mature bursa enters the oviduct, e.g. Helix pomatia, the immature diverticulum clearly arises from the female duct (Jhering, 1875) (Fig. 306a), but in animals whose bursae open into the genital atria the diverticulum branches from the anterior end of the male duct e.g. A.hortensis

(Fig. 306b), the anterior end of the female duct e.g. D.reticulatum, Limax maximus (Hoffmann, 1920) (Fig. 306c) or buds directly from the upper atrium e.g. A.ater (Lüsis,1961) (Fig. 306d). These differences are probably insignificant however, only reflecting slight variations in atrial expansion.

Maturation of the reproductive tract, with the accumulation of secretion in the accessory sex organs, proceeds throughout the spermatozoon, D-E, stages (Lüsis, 1961; Visser, 1973).

ENVIRONMENTAL CONTROL OF REPRODUCTIVE ACTIVITY

The major function of a reproductive system is to regulate the flow and packaging of gametes at copulation and oviposition. To study these events in any detail they must be controlled experimentally and this requires a comprehensive knowledge of the factors affecting reproductive activity in closely related species.

The majority of pulmonates living in a northern, temperate climate have seasonal breeding cycles. These will influence the long term processes of reproduction, i.e. growth and maturation of the gonad and accessory sex organs, but there has to be a more immediate control over reproductive activity which stimulates the short term processes of reproduction, i.e. courtship, copulation and egg-laying, when conditions are optimal. This "fine-tuning" is controlled by a combination of environmental (exogenous) and nervous and hormonal (endogenous) factors.

The experiments were designed following an extensive survey of the relevent literature and from observations on the general reproductive behaviour of animals in the laboratory and in the field.

In the laboratory eggs were laid shortly after collection, following cleaning and feeding, following stress, e.g. inadvertant dehydration or starvation, and at peak periods throughout the year. Copulation, however, was only observed in the field and only on moist, still nights (22.00 - 02.00 hours), since wind or heavy rain limited activity. Egg-laying normally occurs underground and therefore it is seldom seen in the field.

LITERATURE REVIEW

1. Breeding cycles

The activity of slugs is directly related to the environment. Temperature, humidity, food availability and the photoperiod all influence their behaviour, and reproduction is timed to coincide with conditions which favour maximum survival of the young. This results in seasonal fluctuations in slug populations.

Most British species have an annual breeding cycle (Table 3). Eggs are laid during the autumn and winter months but growth of the newly-hatched animals is interrupted until the spring when the climate becomes milder and there is abundant new spring vegetation. Rapid growth and maturation proceeds throughout the summer and by autumn the new adults are ready to mate

Table 3 Annual breeding cycles of common British slugs

	HATCH	GROWTH	MATURATION	EGG LAYING
Arion hortensis[1,2,3]	Jan. - Feb.	Spring - Summer	Sept. - Oct.	Autumn - Winter
A. subfuscus[1,2]	Winter	Spring	July	Aug. - Sept.
A. ater ater[4]	Oct. - Nov.	Spring	July	Aug. - Sept.
A. ater rufus[5]				
Milax sowerbyi[1]	Early Spring	Summer	Sept. - Nov.	Sept. - Nov.
M. budapestensis[1]	Winter	Spring - Summer	Oct. - Nov.	Autumn - Winter
Limax flavus[6]	May	Summer	Nov.	Winter
L. maximus[7]	Dec.	Spring - Summer	♂ phase June - July ♀ phase Sept.	Autumn - Winter

Ref: 1. Bett 1960
2. Barnes and Weil 1945
3. Davies 1977
4. Parivar 1978
5. Laviolette 1954a
6. Takeda 1977
7. Sokolove and McCrone 1978

and lay their eggs. This breeding pattern results in an influx of young animals during the early part of the year, but as maturation proceeds the proportion of adults in the population increases and by the autumn immature animals are rarely seen. The adults usually die soon after egg-laying and there is a decrease in population numbers which persists throughout the winter. In a few species, e.g. Milax gagates, there are two consecutive breeding cycles (Galangau, 1964). The gonad regresses at the end of the first reproductive period and remains inactive throughout the winter. Gametogenesis is resumed in the following spring and death follows the second phase of reproductive activity.

D.reticulatum does not conform to this annual cycle and eggs are laid throughout the year (Runham and Laryea, 1968; Hunter, 1968). Breeding is optimal, however, during the spring and autumn months and large numbers of newly-hatched animals appear at these times (Bett, 1960; Runham & Laryea, 1968; Hunter, 1968). Hunter (1968) observed that these peaks correspond to two successive generations. The spring generation hatch in April to May. They grow rapidly throughout the summer while there is abundant vegetation, and maturation is complete within five months, so that their eggs are laid in the autumn of the same year. These eggs give rise to the autumn generation which hatch in late September to October. They grow more slowly than the

spring hatchlings due to the colder weather and limited availability of food (Bett, 1960), but are fully mature within seven months. They mate and lay their eggs in the spring and summer of the following year and thus give rise to the next spring generation. The adults of both generations die soon after egg-laying.

Despite this twice-yearly trend in D.reticulatum the spring and autumn breeding periods are not well defined and there is a constant population of both adults and young throughout the year. This contrasts greatly to the annual cycle of A.hortensis but in both species the rate of egg production depends very much on the weather conditions, and the total number of eggs produced is very variable.

There are several possible environmental triggers which stimulate egg-laying and which result in the young developing at a time when conditions are most favourable. Changes in the photoperiod and climate are the principal candidates but other factors such as atmospheric pressure, food availability and population density must also be considered.

2. The effect of photoperiod on reproduction

In many species of plant and animal the photoperiod acts as a seasonal cue controlling the annual reproductive cycle. It is a far more reliable predictor of changing seasons than either temperature or humidity and thus ensures that reproductive activity is initiated when food is readily available for maximum growth, and guarantees that seasonal climatic variations, such as winter frosts and summer droughts, are met at a suitable developmental stage capable of surviving the extreme conditions (Menaker, 1971).

Among the terrestrial pulmonates the changing photoperiod plays only a minor direct role in coordinating their behaviour, since these animals spend most of their lives underground where daylength cannot be registered. Despite this, several workers have demonstrated that there is a photoperiodic effect on reproduction. In Limax maximus short day-lengths (8L:16D) suppress the reproductive development of newly-hatched animals (Sokolove and McCrone,1978; McCrone and Sokolove, 1979). Subsequent transition to long days (16L:8D) will stimulate gonad maturation but once development has been initiated it is no longer affected by changes in the photoperiod. From these results Sokolove and McCrone (1978) concluded that maturation of the reproductive tract is triggered by the

annual springtime increase in day-length, and suggested that other external factors, such as temperature and humidity, are more likely to affect the average rate of development than to control its initiation.

Under continuous long-day conditions L.maximus develop normally (Sokolove and McCrone, 1978). Egg-laying commences during the autumn and continues until January when it rapidly declines. This parallels the breeding habits of the wild population. Since the laboratory-reared animals experience constant conditions ($15^{o}C$,16L:8D) there are no environmental cues to stimulate egg production. Sokolove and McCrone(1978) propose that this annual cycle is a reflection of the photoperiodic influence on the original animals which were caught in the wild, and that uniform development maintains this annual rhythm.

Studies on Limax flavus also showed that under constant conditions egg-laying regularly began in early August, reflecting the reproductive cycle of the wild population (Segal, 1959). Separate groups of animals were reared at either 10^{o} or $20^{o}C$ under a continuous light regime of 11L:13D. Although the lower temperature shortened the period of egg-laying it did not alter the timing. This precludes the theory that a fixed length of development maintains the annual cycle of reproduction

and Segal concluded that in these animals an internal clock geared to the solar year regulates the onset of egg-laying.

Smith (1966) was also unable to demonstrate a significant photoperiodic effect on the control of egg-laying in Arion ater . Possibly his experimental animals, collected from the wild, had already been entrained by the natural day-length, so that subsequent changes in the photoperiod would be ineffectual. However, he did show that when A.ater is reared in continuous light (24L:0D) the gonad develops abnormally. This was also observed in D.reticulatum (Henderson and Pelluet, 1960). After five weeks of constant illumination the germinal epithelium appeared thickened and there were large numbers of Sertoli cells. Accelerated meiosis in the male gametes resulted in many multinucleated spermatids and disturbed cytokinesis. Continuous darkness also caused an increase in the number of Sertoli cells but cytokinesis was unaffected. The gonad resumed normal development as soon as the photoperiod returned to more natural levels.

These slugs must be able to detect the changing photoperiod even if it is not the main stimulus for egg-laying. In L.maximus it has been shown that the optic tentacles are not necessary for distinguishing between long and short day-lengths and it is thought

that an extraocular photo-receptor lies somewhere in the central nervous system, since illumination of isolated brains results in increased activity in some of the ganglionic roots (McCrone and Sokolove, 1979). Similar light-sensitive neurons have been postulated in two marine gastropods, the sea hare, Aplysia californica (Brown et al. ,1977; Lickey and Wozniak, 1979) and the limpet, Onchidium verruculatum (Gotow,1975).

One might expect that the photoperiod has a greater direct influence on the breeding behaviour of aquatic pulmonates, since these animals are not obliged to shelter from the desiccating effects of the sun as are the terrestrial species. However, this is not necessarily the case, and although the changing photoperiod can be detected by some species and may play some part in synchronising egg-laying, it is not the principal stimulus for reproductive activity.

In the freshwater basommatophoran, Lymnaea stagnalis, day-length influences both body-growth and egg-production (Bohlken, 1977; Bohlken and Joosse, 1982). Animals reared under short day (8L:16D) or medium day (12L:12D) conditions showed constant growth and reproduction until death, laying, on average, one capsule every four weeks. When kept on long days (16L:8D), however, ovipository activity began 3 to 4 weeks earlier than those exposed to other light regimes

and the rate of egg-laying was maintained at a much higher level, with one capsule being produced every two days. This enhanced egg-production is at the expense of somatic growth and it eventually leads to lethal exhaustion of the animals' reserves.

Earlier studies on L.stagnalis had concluded that the photoperiod had no effect on egg-laying (Steen, 1967). Mature animals had been exposed to either medium day-lengths (12l:12D) or constant illumination (24L:0D) and the quantity and quality of the eggs produced had been recorded. There was no observable difference between animals in the two different light regimes and these conflicting results suggest that egg-laying is not stimulated by a simple light-dark threshold but that it is mediated by other environmental factors.

DeWitt (1967), working on a range of physid and lymnaeid snails, also found that reproductive activity could be enhanced by long day-lengths. In L.pallustris egg-production was initiated at room temperature when the light phase exceeded 13.5 hours, but this could be suppressed by temperature fluctuations, so that when the temperature fell egg-laying ceased, regardless of day-length. Similar behaviour was seen in L.stagnalis and Pomacea paludosa but no such response could be elicited in ~~the closely-related species~~, Physa gyrina, and these animals appear to be unaffected by the

changing photoperiod. Interestingly, DeWitt observed that the nocturnal snail, Physa pomilia, laid most eggs when kept in continuous darkness (0L:24D). In addition, animals reared under these conditions reproduced normally and all commenced egg-laying in September. Thus it seems that although P.pomilia are able to detect day-length, as indicated by their preference for constant dark it is not necessary for synchronizing reproductive activity.

Recent work has now shown that animals which respond to photoperiodic cues are sensitive to short bursts of light during their dark phase. Early experimentors were unaware of this high level of sensitivity and their results may have been affected by inadvertantly exposing their animals to light.

3. The effect of climate on reproduction

Although the photoperiod may play an important part in reproductive maturation it is unlikely to control reproductive behaviour directly, as its effects are generally overridden by the everchanging weather conditions (Segal, 1959; DeWitt, 1967; Sokolove and McCrone, 1978). The weather has an immediate effect on molluscan populations and most species will gradually increase in numbers until severe conditions, such as drought or frost, depletes them. These regular checks

on population growth normally prevent the community becoming too large for the environment which supports it (Solomon, 1969; Runham and Hunter, 1970). This, however merely reflects the gross influence on the population as a whole. Temperature and humidity have direct effects on the reproductive cycles of individuals.

i) Effect of humidity

Evolution to a life on land brought about two major changes, the gills were replaced by lungs and, since external fertilization was now impracticable, the terrestrial gastropods further elaborated ~~developed~~ genital ducts which could convey and receive sperm at copulation and also provide both nutritive and protective layers for the internally fertilized eggs. This move onto land however created problems of dehydration and consequently the higher terrestrial pulmonates have developed both physiological and behavioural adaptations to ensure their maximum survival.

Physiological adaptations

The Helicacea, Arionidae and Limacidae can tolerate considerable fluctuations in their body-water content (Howes and Wells, 1934). Martin et al. ,(1958) estimated that in A.ater around 86% of the total body-weight is water. This can be lost by evaporation

and in the mucus necessary for locomotion. Therefore, even in a saturated atmosphere any activity will result in some loss of water. If this falls by approximately 25% the animals become inactive and so minimize further dehydration. This also holds true for D.reticulatum and L.maximus, and all three species have been known to survive a 50% decrease in their body-water content (Dainton, 1954; Runham and Hunter, 1970). Dainton (1954) has shown that in L.maximus this deficit can be rectified within two hours, by absorbing atmospheric moisture through the skin.

A calcareous shell protects the snails from injury and desiccation, and, in addition, Helix aspersa can regulate water loss from the mantle edge (Machin, 1966). The slugs, however, have no structural protection from, and little physiological control over, dehydration. They must adopt a pattern of behaviour which minimizes the risks of desiccation.

Behavioural adaptations

During the day slugs are inactive and shelter beneath stones and rotting vegetation. They frequently burrow down into the ground, travelling rapidly along worm tunnels (Alan Carpenter, pers. comm.) where they are well protected from the drying effects of the sun and wind. They generally remain in the top 10cms. of

soil, but winter frosts and prolonged summer drought will force them to go deeper, to warmer, wetter layers.

Slugs are most common in heavy soils which have a high water content throughout the year. Carrick (1942) has shown experimentally that D.reticulatum can quite accurately assess the soil condition and will preferentially congregate in soils at 64% saturation. Ground cover also influences their distribution since it provides both shelter and food, as well as reducing the amount of water lost from the soil by evaporation. Consequently, wherever the vegetation is sparse, slug populations are small (Runham and Hunter, 1970).

The slugs emerge in the early evening when the ambient temperature begins to fall and the humidity at ground level increases. Barnes and Weil (1945) reported that there was maximum activity on warm still nights, when there was plenty of surface moisture in the form of dew or recently fallen rain. This agrees with our own observations on D.reticulatum and A.hortensis and under such conditions slugs of all sizes may be found on open grassland, including newly-hatched animals which are particularly vulnerable to desiccation. Freezing temperatures, lack of surface water, heavy rain and wind all limit activity and only the largest and toughest of the species will venture out.

Activity is maximal between dusk and midnight, but then steadily decreases so that by dawn few slugs are out on the surface (White, 1959). This diurnal rhythm arises from the necessity to avoid sunlight and is reinforced by a phototactic response (Lewis, 1969). The optic tentacles are sensitive to light intensity. During the day the slugs display negative phototaxis and actively seek out shady, sheltered spots. At night, however, this response alters and the animals become positively phototactic choosing weak illumination in favour of total darkness. Thus, bright moonlight does not inhibit activity. Lewis suggests that this response regulates the onset of activity at dusk, the long, summer evenings keeping the slugs underground, whilst dull, cloudy days would encourage them to emerge earlier than usual.

Newell, observing the nocturnal behaviour of slugs, could find no overall pattern in the occurrence or duration of the different activities (Newell, 1966; Newell, 1968). Irregular periods of locomotory, feeding and mating activity are interspersed with periods when the animals seem to be resting. Specific stimuli for these activities could not be identified although the weather conditions were found to have a direct influence on their intensity. Locomotory and feeding behaviour can be regulated to suit the situation, so that when conditions are poor activity is minimal. Extended

courtship rituals, however, expose the slugs to the elements for long periods and copious mucus is produced, particularly during sperm transfer. This suggests that copulation is expensive in terms of water loss and is only observed in the wild when conditions are optimal. Egg-laying is rarely witnessed, since this usually occurs underground.

The eggs have a poor capacity for retaining water and there is no structural provision for resisting desiccation (Bayne, 1968). Developing embryos are most vulnerable in newly laid eggs (Bayne, 1969) and it is essential for their survival that the slug selects a suitably moist site for ovipositiion. Experiments on D.reticulatum demonstrated that these animals preferentially lay their eggs in soils at 50 to 75% saturation (Carrick, 1942; Arias and Crowell, 1963). They completely reject soils if the saturation level is less than 10% and batches laid at 25% and 100% fail to develop. In the wild the depth of oviposition varies with the moisture content and so the drier the soil, the deeper the eggs are laid (Carrick, 1942).

The eggs remain underground and untended until they hatch. During this time the condition of the soil can alter and soils which were suitable at egg-laying may dry out or become waterlogged. The effect on the eggs however decreases with age, and as the embryos

develop they become increasingly tolerant to both desiccation and flooding. The eggs of D.reticulatum and Limax flavus can survive short-term water losses of up to 80% (Bayne, 1968) while complete submersion for four days has no effect on development (Arias and Crowell, 1963).

These observations clearly demonstrate that slug activity is greatly influenced by both the relative humidity of the atmosphere and the moisture content of the immediate environment. Relative humidity is directly related to temperature, and excessively high or low temperatures affect the availability of free water, either by evaporation or by freezing. Temperature, therefore, is a key factor in regulating behaviour, influencing the slugs directly, as well as indirectly via their dependence on water.

ii) Effect of temperature

Dainton (1954) claims that slugs are very sensitive to fluctuations in temperature and D. reticulatum can perceive changes of only 0.1^{o}C per hour. Such sensitivity enables the animals to respond rapidly to very slight variations in the immediate environment. This response varies according to whether the change in temperature is favourable or not. Falling temperatures

in a cold climate and rising temperatures in an already warm environment both induce locomotory activity, which directs the slug away from potentially adverse conditions (Dainton, 1954; Arias and Crowell, 1963).

Slugs are normally active between 4°C and 25°C (Carrick, 1942; Barnes and Weil, 1945). D.reticulatum and A.hortensis are the two species most resistant to cold, and locomotory activity in these animals may continue down to freezing-point (Barnes and Weil, 1945; White, 1959). There is no movement below 0°C and most animals are killed by temperatures of less than -3°C (Runham and Hunter, 1970). Excessively high temperatures (>30°C) also inhibit activity. Such conditions invariably result in death.

Reproductive activity is restricted to a much narrower range of temperatures (10°-20°C) than either feeding or locomotion (4°-25°C) (Carrick, 1942; Arias and Crowell, 1963). This serves two purposes. Mating pairs remain on the surface for long periods while they perform their lengthy courtship rituals prior to copulation. Courtship may last for up to two hours and during this time the animals are completely unprotected from the environment. Consequently it is advantageous if this behaviour is restricted to times when the

climatic conditions are optimal. Secondly, newly-laid eggs are particularly vulnerable to extremes of temperature and rarely survive freezing or temperatures greater than 22°C (Carrick, 1942; Arias and Crowell, 1963). Therefore, to ensure maximum survival of their young, oviposition only proceeds if the temperatures are favourable for development. Resistance increases with age, so if the climate subsequently deteriorates the eggs are less likely to perish, and as development nears completion the embryos can withstand freezing temperatures for several days (Carrick, 1942; Arias and Crowell, 1963).

The temperature continually influences the rate of embryonic development and therefore the length of incubation (Carrick, 1938); 1942). In D.reticulatum development is optimal at 15°C and hatching occurs 25 to 36 days after the eggs are laid. This is not the minimal period for development, however. At 20°C incubation may last for only 15 days, but higher temperatures encourage parasitic infections and the mortality rate increases exponentially. Eggs incubated at temperatures greater

than 22°C all fail to hatch. As the temperature falls the period of incubation lengthens, and at 5°C it can take up to 118 days for the embryos to fully develop. Although the risks of infection are minimal at these low temperatures survival decreases with lengthening incubation and at 0°C hatching is completely inhibited (Carrick, 1938; 1942). A similar response to temperature has been observed in A.ater rufus (Laviolette, 1954 a), Milax budapestensis (Runham and Hunter, 1970) and A.hortensis (Davies, 1977).

From his observations Carrick successfully stimulated hatching in the laboratory. Fully-developed eggs were kept at -2°C for two hours and then returned to more favourable temperatures. All started hatching within a few hours, whereas eggs left unfrozen did not begin to hatch until two days later. This suggests that hatching is induced by changes in temperature.

Temperature continues to affect development after hatching and low temperatures will delay maturation and therefore increase the length of time between generations (Carrick, 1942; Arias and Crowell, 1963; Runham and Hunter, 1970).

The reproductive activity of aquatic pulmonates is also affected by the climate, but water considerations are, of course, unnecessary and temperature is the principal factor controlling both mating and egg-laying. Reproduction is inhibited in cold waters but is resumed when the temperature rises to more favourable levels (Boerger, 1975; DeWitt, 1967). In constantly warm waters Radix peregra (Krkač, 1979) and Physa gyrina (DeWitt, 1967) can breed all year round, but for most species changes in the environment regulate behaviour. A steady increase in water temperature will stimulate oviposition in Lymnaea stagnalis (Timmermans, 1959; DeWitt, 1967), L.pallustris (Cheatum, 1951), Planorbis corneus and P.planorbis (Timmermans, 1959), but has no effect if the temperature exceeds 35°C. Since temperature affects the amount of oxygen dissolved in the water, these factors probably interact to influence egg-laying. Thus, sunlight and rising temperatures encourage photosynthesis, and the oxygen levels increase wherever there is abundant vegetation. If the water becomes too warm, however, the oxygen comes out of solution and the stimulus disappears.

In L.stagnalis changes in the atmospheric pressure can induce egg-laying (Steen, 1967); but it is unlikely that pressure fluctuations directly influence this activity. Since pressure also affects oxygenation of

water, increasing oxygen levels may be the actual stimulus for oviposition. Janse et al. ,(1982) have now shown that in L.stagnalis the oxygen levels in the water are detected by sensory cells which are mainly located in the mantle wall.

The effect of humidity and temperature on oviposition may not always be an immediate response to the changing environment. Suppression and enhancement of egg-laying can arise from a long-term influence on gametogenesis.

Bouillon (1956) first observed that temperature affected gonad development in Cepaea nemoralis. High temperatures (23°C) increase the rate of spermatogenesis at the expense of oocyte development. Low temperatures have the reverse effect, and at 6°C differentiation of the male gametes is delayed or suppressed, whilst oogenesis proceeds normally. Gametogenesis is completely inhibited when the temperature falls below 0°C. Subsequently Joosse (1964) and Guyard (1971), working on Lymnaea stagnalis and Helix aspersa respectively, observed that sperm development is blocked during the winter, due to low environmental temperatures of

less than 10°C. Providing these remain above freezing, however, oogenesis appears to be unaffected.

Differentiation of the slug gonad is similarly affected by the environment. In Arion ater high temperatures disrupt the development of both male and female gametes, but they are particularly damaging to the maturing oocytes (Lüsis, 1966; Smith, 1966). Low temperatures however favour oogenesis by delaying sperm differentiation, and abnormal spermatogonia and degenerating spermatocytes appear in the gonad (Lüsis, 1966; Smith, 1966; Parivar, 1978). Again, freezing conditions completely suppress gamete development.

Parivar (1978) and Lüsis (1966) both suggest that humidity also influences gametogenesis in A.ater. Parivar found that high relative humidities (65 - 95%) encouraged oocyte development whilst adversely affecting spermatogensis. Lüsis, on the other hand, reports that low humidities favour differentiation of the oocytes. These results do not necessarily preclude one another and abnormally high and low humidities may both suppress spermatogenesis and therefore favour oocyte development. These experiments, however, did not exclude all other environmental variables. Gamete differentiation may have been influenced by temperature alone, and more

conclusive evidence is needed before the effect of humidity on gametogenesis can be confirmed.

The response of the gonad to prolonged, adverse, weather conditions guarantees that gametogenesis does not proceed when reproductive activity is suppressed. This effectively conserves resources until conditions become favourable once more. Oogenesis, however, appears to be more resistant to low temperatures than spermatogenesis and changes in the rate of sperm differentiation alter the ratio of male to female gametes. In winter, therefore, the oocytes differentiate preferentially and this possibly prepares the animal for when egg-laying commences in the Spring.

4. The effect of the quantity and quality of food on reproduction

Coe (1944) observed that an abundance of food encouraged egg-laying in a variety of molluscs, ranging from the primitive Amphineura to the most advanced Cephalopoda, and in situations where the food source might fluctuate it is obviously advantageous to breed when there are sufficient supplies to support the young.

In L.stagnalis, if food is supplied in excess, the level of consumption increases and more eggs are produced (Steen et al. , 1973). Starvation has the

reverse effect, leading to cessation of egg-production and the eventual resorption of the gonad (Joosse, 1979). In Arion ater starvation during the growth period delays reproductive maturation for a whole year (Chevallier, 1971). Recent research on L. stagnalis has shown that the number of eggs produced is directly proportional to the amount eaten (Scheerboom, 1978) and Scheerboom further demonstrated that a critical minimum amount of food is necessary for normal growth, i.e. 5mg lettuce/day, but for reproduction this must increase to 6-7mg daily. Veldhuijzen and Cuperus (1976) proposed that the levels of glucose in the haemolymph acted upon endocrine centres, which in turn regulated reproductive activity. Raising the level of circulating glucose, experimentally, inhibited feeding, although growth and egg-laying were unaffected (Scheerboom and Doderer, 1978). Since the level of food consumption is critical for both these processes it appears that the concentration of haemolymph glucose regulates both growth and reproduction.

The quality of food also affects fecundity. Eisenberg (1970) found that the addition of only a few milligrams of spinach to the daily diet of Lymnaea elodes increased the average clutch size, while Scheerboom (1978) observed that L.stagnalis fed on Bemax produced more eggs than those fed on a similar amount of lettuce. In Biomphalaria glabrata vitamin E stimulates

egg-production (Viera, 1967) and Viera suggests that seasonal fluctuations in production reflect the changing quality of the food. This is seen in farm livestock, where the digestible organic content of the grass decreases during the early summer and coincides with a reduction in reproductive productivity (Spedding, 1971). In Cepaea nemoralis, Wolda and Kreulen (1973) noticed a similar, seasonal decrease in clutch-size which was unrelated to age or temperature. After completion of a clutch the albumen gland was empty and ovulated eggs remained in the fertilization chamber. Wolda and Kreulen suggest that the clutch size is determined by the capacity of the albumen gland rather than the number of eggs ovulated. Since the quantity of food available does not vary, they conclude that a change in quality affects the snails' ability to synthesize the protein and galactogen components of albumen.

Oviposition can be stimulated experimentally by supplying animals with their preferred food after a period of deprivation (Timmermans, 1959). If L.stagnalis are kept on a diet of lettuce but are then fed on the leaves of Hydrocharis morsus ranae and Trianea bogartensis the rate of laying increases dramatically. Similarly, the water plant Ludwigia stimulates oviposition in Australobis glabratus within 24 hours. These responses, however, could be due to

increased oxygen levels in the water, which would result from the addition of oxygenating plants.

5. The effect of population density on reproduction

The density of a population directly affects the amount of food available and, in the case of the aquatic pulmonates, the oxygen levels of the water, both of which, will in turn, affect reproduction. Laboratory-reared L.stagnalis produce fewer egg-capsules as the density increases (Mooij-Vogelaar et al. , 1973). When the food supplies are sufficient to maintain the population the limiting factor appears to be the increased oxygen requirement. Alternatively, the snails may release a hormone which suppresses ovulation or oviposition, its concentration in the water increasing as the population grows (Thomas and Benjamin, 1974).

In Physa gyrina and Pseudosuccinea columella egg-laying can be enhanced by rearing the animals in isolation (DeWitt, 1967). This response maximizes the reproductive potential of an individual when it has found a new, unpopulated site and will help to establish a new colony. Physa pomilia on the other hand takes longer to mature and lays fewer, less-viable eggs when isolated. DeWitt suggests that in this species copulation is important for stimulating oviposition.

The density of slug populations has little effect on the food supply, which usually far exceeds their basic requirements. Competition for suitable shelter, however, leads to inter- and intra- specific aggression amongst certain species, e.g. Limax maximus, Arion subfuscus and Deroceras caruanae (Rollo and Wellington, 1980). Those shelters closest to food supplies are in greatest demand, since travelling depletes water reserves. Nearly all the slugs observed laid their eggs inside these shelters and therefore favourable sites for the adults also favour the offspring. A shortage of suitable shelter-foraging areas, therefore, may effect some degree of population control, but population density does not appear to have a direct influence on reproductive capacity.

6. The endogenous control of reproduction and its relationship with the environment

When attempting to assess the effect of the environment on reproduction experimental limitations make it necessary to treat each environmental or exogenous factor separately. Breeding cycles, photoperiod, temperature, humidity, food availability and population density however are all interrelated and their combined affects on reproductive behaviour must be considered in relationship with the hormonal or endogenous influence on reproduction.

Amongst the pulmonates, most of the work on the endocrine control of reproduction has been carried out on the freshwater basommatophoran, L.stagnalis. In this animal female reproductive activity is controlled by two cerebral endocrine centres, the dorsal bodies (DB) and the caudo-dorsal cells (CDC). The DB produce a hormone (DBH) which stimulates vitellogenesis and differentiation and activity of the female accessory sex organs (ASO) (Geraerts and Joosse, 1975; Geraerts and Algera, 1976; Wijdenes et al. , 1983; Joosse and Geraerts, 1983). The neurosecretory CDC release an ovulation hormone (CDCH) from axon terminals located in the cerebral commissure (Geraerts and Bohlken, 1976; Geraerts et al. , 1983). Small ganglia attached to the cerebral ganglia, known as the lateral lobes (LL) control female activity indirectly, by activating the DB-cells and CDC (Geraerts, 1976; Roubos et al. , 1980).

Field animals show an annual cycle of DB and CDC activity which coincides with oviposition (Joosse, 1964). In addition, the CDC exhibit a physiological cycle related to the formation of egg masses (Kits, 1980). Environmental factors exert their effects on reproduction via extra-ocular receptors which transmit information to the neuroendocrine system (Minnen and Reichelt, 1980; Dogterom et al. , 1983) and have a direct effect on the activities of the CDC and possibly the DB (Minnen and Reichelt, 1980; Scheerboom, 1978;

Scheerboom and Doderer, 1978). This activity may be regulated by the LL.

Endocrine control of reproduction in the stylommatophoran pulmonates is less clearly understood. The cerebral ganglia control gametogenesis and growth and maturation of the ASO, and their activity is directly related to the photoperiod (Guyard, 1971; Sokolove and McCrone, 1978; McCrone and Sokolove, 1979). Spermatogenesis and male ASO development are under their direct influence, but oogenesis and the development of the female ASO are mediated by the DB-cells (Wijdenes and Runham, 1976). Cells, functionally analogous to the CDC have been found in only one terrestrial pulmonate, Helix aspersa (Wijdenes et al. , 1980), and to date no area comparable to the LL has been indentified.

In contrast to the Basommatophora, the optic tentacles and the gonad of the Stylommatophora have endocrine functions (Pelluet and Lane, 1961; Runham et al. , 1973). In A.subfuscus Wattez (1973; 1975; 1978) has demonstrated that the optic tentacles contain and release an androgenic factor(s) which stimulates spermatogenesis and inhibits autodifferentiation of the oocytes. This factor(s) is probably present in other terrestrial pulmonates. The gonad appears to have dual control of ASO development (Runham et al. , 1973), one hormone controlling differentiation and enlargement of

the prostate gland, and the other controlling growth and differentiation of the female ASO. No androgenic factor has been found in the basommatophorans.

Joosse and Geraerts (1983) suggest that this disparity in the control of reproduction is directly related to the type of hermaphroditism commonly seen in these two pulmonate orders. In the Basommatophora, where simultaneous hermaphroditism is preceded by only a brief protandric period, the start of sexual activity is controlled by one centre, the LL, which stimulates both sexes. In the Stylommatophora, however, where there are separate male and female phases the onset of reproductive activity is controlled by a factor which stimulates the male, and inhibits the female line. The release of this factor decreases as the animal matures.

THE EFFECT OF PHOTOPERIOD ON MATURATION AND REPRODUCTIVE ACTIVITY

From the literature, it appears that the photoperiod is more important in controlling the onset of maturation in young slugs than in stimulating reproductive behaviour in fully mature adults. It was necessary to see if this was true for D.reticulatum and A.hortensis.

Materials and methods

When rearing slugs in the laboratory the environment was adjusted so that conditions were optimal for both growth and reproduction. Stock animals were kept under a photoperiod that results in a breeding cycle typical of animals in the field. The temperature was maintained at 15°C. Humidity was difficult to control accurately in large volumes and the animals were placed in sandwich boxes with closely fitting lids. Moistened cellulose wadding provided constant high humidity and shelter. The atmosphere of such an enclosed environment soon became stale and it was necessary to clean and aerate the slugs every other day. Food, in the form of lettuce, "Tetramin" fish food and muesli base, was supplied ad libitum.

In addition to the natural daylight conditions of the animal house, two other light regimes were established, one corresponding to a long-day cycle (16L: 8D) and the other to short days (8L:16D). In all other respects the animals were maintained as the stock animals.

i) The effect of photoperiod on growth

Eggs laid by stock D.reticulatum and A.hortensis were incubated in the animal house. They hatched in mid-September and the young slugs were transferred immediately to the three different light regimes. Since the constitution and rate of development of the young varies between batches, each brood of hatchlings was divided equally between the three environments. Each group finally contained 30 to 35 animals.

Newly-hatched slugs are too small and delicate to be handled individually on a regular basis and for this reason they were weighed as a group until 4 weeks old. The average weight for each group was recorded at the start of the experiment and then at 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12 weeks. The difference in growth between animals reared in daylight and those reared under long- or short-day regimes were compared using the t-test for uncorrelated (unpaired) samples, and expressed in terms

of probability (P), taking the conventional significance level of 5 per cent (0.05).

ii) The effect of photoperiod on gonadal development

Eggs laid by stock D.reticulatum and A.hortensis were incubated in the animal house. They hatched throughout May and the young slugs were transferred immediately to the three different light regimes, as before. Once the groups experiencing artificial light contained 30 to 35 animals, all subsequent hatchlings were reared in natural daylight.

Individuals were sampled at regular intervals after hatching and their gonads prepared for light microscopy.

The age of each animal was correlated to the stage of the gonad using the classification described by Runham and Laryea (1968).

iii) The effect of photoperiod on egg-production

Adult D.reticulatum and A.hortensis were collected from the wild and divided equally between the three different light regimes. Each group contained 30 to 40 animals, and as the experiment progressed new slugs were added to replace post-reproductive or moribund

individuals. In this way the population of mature, egg-laying animals was maintained. A period of one month was allowed for acclimatization.

Each batch of eggs was counted. The average clutch size and the total number of eggs produced were then calculated monthly.

Individual laying patterns were observed by isolating slugs in specially partitioned boxes. Gauze screens divided the boxes into six compartments and this kept the egg-batches separate whilst still allowing limited tactile, optic and olfactory contact. Groups of six animals were placed in each of the three different light regimes and egg-production was monitored.

iv) <u>The effect of photoperiod on incubation and fertility</u>

The eggs laid by the animals in (iii) were incubated under their appropriate light regime. The clutches were isolated in petri dishes, kept damp with moistened cellulose wadding, and the eggs in each clutch were counted.

The incubation period was measured from the date of laying to the day the first egg hatched. Eggs which had failed to hatch after 60 days were discarded.

v) Abnormal development of A.hortensis

During the course of these experiments it was noticed that whilst D.reticulatum bred normally in captivity, with three successive generations of slugs laying viable eggs, the second generation A.hortensis were sterile.

To investigate this the eggs of wild A.hortensis were incubated in the animal house and the newly-hatched animals reared entirely under laboratory conditions. These first generation animals matured normally (see (ii)) and produced large numbers of fertile eggs. The second generation of slugs hatching from these eggs was sampled at regular intervals after hatching. Their reproductive tracts were prepared for light microscopy and the gonads staged using the classification described by Runham and Laryea (1968).

Results

i) The effect of photoperiod on growth

The growth rates of both D.reticulatum and A. hortensis were influenced by the photoperiod and the animals grew most rapidly when reared under a long-day cycle (Table 4; Fig. 307).

Table 4a

The effect of photoperiod on growth

D. reticulatum											
	DAYLIGHT			16L:8D				8L:16D			
WEEKS	Av. Wt.	Max.	Min.	Av. wt.	Max.	Min.	P	Av. Wt.	Max.	Min.	P
0	4.1	-	-	4.0	-	-	-	3.8	-	-	-
1	4.5	-	-	4.6	-	-	-	4.4	-	-	-
2	14.5	-	-	14.8	-	-	-	14.7	-	-	-
3	28.6	-	-	34.7	-	-	-	29.8	-	-	-
4	43.1	45.7	38.0	55.6	63.2	50.9	<0.001	42.9	47.2	39.7	>0.25
5	90.5	96.4	87.6	120.9	126.2	113.6	<0.001	87.6	92.0	84.2	>0.25
6	145.8	151.5	140.1	186.2	193.5	179.9	<0.001	141.1	146.7	136.5	>0.25
7(+1day)	184.3	190.0	175.3	225.3	237.2	216.4	<0.001	179.1	187.1	171.6	>0.25
8	230.1	242.0	221.7	260.0	278.2	245.5	<0.001	224.0	234.7	211.9	>0.25
10	268.4	281.8	243.3	333.6	360.4	322.7	<0.001	256.3	275.0	244.1	0.25-0.1
12	299.0	329.4	262.4	394.9	410.4	375.3	<0.001	280.2	301.7	250.5	<0.001

P >0.05 not significant
P <0.05 significant difference

Weights in milligrams

Table 4b The effect of photoperiod on growth

A. hortensis											
	DAYLIGHT			16L:8D				8L:16D			
WEEKS	Av. Wt.	Max.	Min.	Av. Wt.	Max	Min.	P	Av. Wt.	Max.	Min.	P
0	4.9	-	-	4.8	-	-	-	4.8	-	-	-
1	5.3	-	-	5.1	-	-	-	5.3	-	-	-
2	14.0	-	-	13.7	-	-	-	12.9	-	-	-
3	22.4	-	-	25.8	-	-	-	21.6	-	-	-
4	32.1	34.9	29.3	36.4	39.8	32.1	>0.25	33.3	37.2	30.0	>0.25
5	44.9	48.4	40.4	53.9	58.9	47.8	0.25-0.1	42.2	47.9	37.6	>0.25
6	82.8	89.2	75.5	87.4	98.3	80.9	0.05-0.02	73.6	80.1	65.1	0.25-0.1
7(+1day)	105.6	115.9	96.7	148.5	162.3	139.0	<0.001	100.1	114.8	92.9	>0.25
8	168.6	182.5	156.4	217.2	229.9	201.3	<0.001	144.7	157.5	127.6	<0.002
10	202.0	226.5	186.7	272.0	297.3	255.6	<0.001	195.9	218.1	173.1	0.25-0.1
12	249.2	261.3	206.8	319.1	338.6	285.0	<0.001	242.2	266.1	219.9	>0.25

P >0.05 not significant
P <0.05 significant difference

Weights in milligrams

The effects of the different light regimes were first observed after three weeks when the groups experiencing long daylengths began to show greater increases in body weight. This enhanced rate of growth was maintained throughout the experiment and after twelve weeks the 'long-day' animals were 30-40% heavier than those of the other two groups.

Growth is not completely suppressed by short daylengths and the animals show a steady increase in weight over the twelve week period.

From mid-September to mid-December the natural daylight is steadily decreasing and for most of the time the group reared in the animal house experience short day-lengths. Not surprisingly these animals grow at a similar rate to those kept on the short-day regime, but they tend to be slightly heavier after five to six weeks. This advantage in size persists and may be due to the longer daylengths experienced at the start of the experiment.

ii) The effect of photoperiod on gonad development

D.reticulatum and A.hortensis show similar rates of gonad development and this is influenced by the photoperiod (Table 5).

Table 5a The effect of photoperiod on gonadal development

D. reticulatum

AGE	STAGE							TOTAL	
	A	B	C	D	E	F/G	H		
1 DAY	4							4	
1 WEEK	3	4						7	
2 WEEKS		7						7	Daylight
4 WEEKS		7	2					9	
6 WEEKS		2	6	2				10	
8 WEEKS		1	3	4	2			10	
12 WEEKS				3	6			10	
18 WEEKS				1	1	2	6	10	

AGE	STAGE							TOTAL	
	A	B	C	D	E	F/G	H		
1 WEEK	2	3						5	
2 WEEKS		4						4	
4 WEEKS		2	3					5	16L:8D
8 WEEKS			1	4				5	
12 WEEKS				2	3			5	
18 WEEKS					1	3	3	7	

AGE	STAGE							TOTAL	
	A	B	C	D	E	F/G	H		
1 WEEK	2	3						5	
2 WEEKS		5						5	
4 WEEKS		4	1					5	
8 WEEKS		1	3					4	8L:16D
12 WEEKS			4					4	
18 WEEKS				1				1	

Table 5b The effect of photoperiod on gonadal development

A. hortensis

AGE	STAGE							TOTAL
	A	B	C	D	E	F/G	H	
1 DAY	3							3
1 WEEK	2	4						6
2 WEEKS		7						7
4 WEEKS		3	5					8
6 WEEKS		2	5	1				8
8 WEEKS			1	5	3			9
12 WEEKS				2	4	2		8
18 WEEKS					1	1	4	6

Daylight

AGE	STAGE							TOTAL
	A	B	C	D	E	F/G	H	
1 WEEK	1	4						5
2 WEEKS		5						5
4 WEEKS		2	2					4
8 WEEKS			2	3				5
12 WEEKS				3	2			5
18 WEEKS					1	3	2	6

16L:8D

AGE	STAGE							TOTAL
	A	B	C	D	E	F/G	H	
1 WEEK	3	2						5
2 WEEKS		5						5
4 WEEKS		3	1					4
8 WEEKS			4					4
12 WEEKS		1	3					4
18 WEEKS								0

8L:16D

Both species mature more rapidly when reared on long daylengths or under the natural daylight conditions of early summer. The gonad of a newly-hatched animal is just a small undifferentiated mass of cells (Stage A). Differentiation begins during the first week and by the end of the second week all the animals contain recognisable spermatogonia, spermatocytes and Sertoli cells (Stage B). Some individuals remain at this level of development until they are six to eight weeks old, while others may enter the spermatid or C-stage as early as their fourth week. Six weeks after hatching the first D-stage gonads are observed, characterized by the newly differentiated spermatozoa which remain closely associated with the Sertoli cells. This stage, however, is most commonly seen when the animals are eight weeks old.

By twelve weeks the gonads of most slugs have reached the E-stage of development. Maturation of the spermatozoa has increased and sperm are released in large quantities into the hermaphrodite duct, which acts as a seminal vesicle. Some twelve week animals have large oocytes present. These may be in their follicles, attached to the acinar wall (Stage F) or free in the lumen (Stage G), and the two stages are difficult to distinguish.

After 18 weeks nearly all the animals sampled appeared to have a thickened cuboidal epithelium forming some part of the acinar wall. This is characteristic of the H- or post-reproductive stage, but since these animals are still capable of laying viable eggs, this would seem to be a misnomer.

Short day-lengths tend to suppress development and after twelve weeks gonad maturation had only progressed as far as stage C. There was higher mortality under this light regime and just one individual D.reticulatum survived to 18 weeks. Gonadal development in this animal had reached stage D.

iii) The effect of photoperiod on egg-production

The photoperiod influenced egg-production in D.reticulatum and A.hortensis, and both species showed regular seasonal variations in egg-laying activity (Table 6; Fig. 308).

a) D.reticulatum

Under the natural light conditions of the animal house D.reticulatum laid most eggs throughout the spring (March to June) and autumn (August to October) (Fig. 308a). Production fell during the summer and winter

Table 6a The effect of photoperiod on egg-production in D.reticulatum

		M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	TOTAL
No. of clutches	Daylight	25	8	8	16	30	28	10	9	4	8	26	30	19	12	9	9	19	21	3	5	299
	16L:8D	-	-	15	21	38	25	23	19	14	14	16	14	9	10	6	7	12	5	5	5	258
	8L:16D	-	-	3	9	8	16	5	1	6	2	3	8	1	0	0	2	0	1	1	0	66
No. of eggs	Daylight	627	192	168	432	742	668	254	239	71	179	607	742	496	330	211	228	463	512	46	75	7282
	16L:8D	-	-	353	526	879	581	568	480	312	354	410	349	208	231	132	183	280	114	101	84	6145
	8L:16D	-	-	56	213	193	354	110	22	108	47	55	167	18	0	0	37	0	7	21	0	1408
Average clutch size	Daylight	25	24	21	27	25	24	25	27	18	22	23	25	26	28	23	25	24	24	15	15	-
	16L:8D	-	-	24	25	23	23	25	25	22	25	26	25	23	23	22	26	23	23	20	17	-
	8L:16D	-	-	19	24	24	22	22	22	18	24	18	21	18	0	0	19	0	7	21	0	-

Table 6b The effect of photoperiod on egg-production in A. hortensis

		M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	TOTAL
No. of clutches	Daylight	2	1	3	21	34	30	14	16	3	5	2	6	5	2	2	9	18	19	10	7	209
	16L:8D	-	-	6	16	20	10	6	1	2	1	1	3	1	3	5	7	10	8	8	5	113
	8L:16D	-	-	6	17	27	30	22	13	16	14	9	1	4	3	2	4	8	15	7	4	202
No. of eggs	Daylight	54	26	90	546	1021	953	448	547	75	140	56	132	141	57	43	283	611	583	297	193	6296
	16L:8D	-	-	203	582	658	329	126	17	59	25	3	96	31	93	146	211	292	198	182	154	3405
	8L:16D	-	-	169	431	887	917	760	397	371	308	244	22	116	74	59	76	241	434	186	105	5797
Average clutch size	Daylight	27	26	30	26	30	32	32	34	25	28	28	22	28	29	22	31	34	31	30	28	-
	16L:8D	-	-	34	36	33	33	21	17	30	25	3	32	31	31	29	30	29	25	23	31	-
	8L:16D	-	-	28	25	33	31	35	31	23	22	27	22	29	25	30	19	30	29	27	26	-

months and egg-laying reached its lowest level in midwinter.

Under constant conditions egg-production deteriorated. Continuous long daylengths caused an initial surge in egg-laying which corresponded to the natural autumnal peak. Production then steadily decreased. There was no sudden winter slump in activity and the number of eggs produced under long day conditions was at first higher than if the animals had been kept in natural daylight, but since production only increased slightly during the spring and autumn, this advantage was soon lost.

Short daylengths further suppressed ovipository activity and the initial peak in production was significantly reduced ($0.02>P>0.01$). A slight increase in the number of eggs deposited during April coincided with the natural rhythm of activity, but as egg-laying became intermittent it was impossible to detect any further seasonal trends. In the majority of animals prolonged exposure to short days had an inhibitory effect on egg-laying.

Clutch size was also affected by the photoperiod (Table 6a). Under natural light conditions each slug produced, on average, six to eight batches of eggs. At first these batches were large, normally containing

between 23 and 28 eggs (max. 38), but as the animals neared the end of their reproductive life the clutches became smaller, until egg-production ceased completely. The average clutch size also fell during periods of reduced ovipository activity, regardless of the animals' age. Consequently small clutches of 10-20 eggs were more common in summer and midwinter.

Long daylengths did not affect the size of the egg batches but the group of animals reared on short days regularly produced small clutches of less than 20 eggs.

b) A.hortensis

In A.hortensis egg-laying was confined to the autumn and winter months (Fig. 308b). Under natural light conditions production rose rapidly during August and reached a peak in September and October. The number of eggs then gradually decreased and ovipository activity was minimal between January and July. During the second year of the experiment fewer eggs were laid in this peak period.

Under constant conditions the annual rhythm of activity deteriorated. Continuous short daylengths produced an initial burst of egg-laying activity, similar to that seen in the animal house, but the rate of laying decreased more steadily. The next annual peak

in egg-laying was considerably reduced, however. Production did not begin to increase until September and fell off rapidly within two months.

Long daylengths further suppressed egg-production although a seasonal trend was still recognisable after one year.

The clutch size did not appear to be affected by the photoperiod (Table 6b). In natural daylight each slug produced, on average, five to eight batches of eggs. The early clutches were always larger, usually containing between 25 and 31 eggs (max. 49), but this decreased as the animals aged until egg-production ceased completely. Unlike D.reticulatum there were no seasonal variations in the size of the egg batches and the groups of animals reared on either long- or short-day regimes showed no apparent difference in the average clutch size.

iv) The effect of photoperiod on incubation and fertility

The photoperiod had no affect on the length of incubation but the clutches laid by animals kept in unfavourable light regimes were less fertile and fewer eggs hatched successfully (Table 7; Fig. 309).

Table 7 The effect of photoperiod on incubation and fertility

		Av. incubation period (days)	Total no. of clutches	Total no. hatched	No. failed to hatch	% infertile
D.reticulatum	Daylight	30	299	272	27	9.0%
	16L:8D	30	258	203	55	21.3%
	8L:16D	30	66	34	32	48.5%
A.hortensis	Daylight	31	209	196	13	6.2%
	16L:8D	30	109	78	31	28.4%
	8L:16D	31	202	163	39	19.3%

In D.reticulatum the minimum incubation period was only 15 days. Such rapid development was rare, however, and most clutches did not begin to hatch until 26 to35 days after laying (av. 30 days). Eggs which had failed to hatch after 45 days were either infertile or had died.

In natural daylight 9% of clutches were completely infertile and of the fertile clutches 8Q to 90% of the eggs hatched successfully. Eggs laid under the long day regime were equally successful until the start of the second year but as clutch production fell, infertility increased, and by the end of the experiment 21% of the clutches were infertile. Short daylengths reduced fertility still further and only half the clutches produced viable offspring.

In A.hortensis the eggs started to hatch after 23 days but for most clutches the first hatchlings did not emerge until 28 to 32 days after laying (av. 31 days). Occasionally a batch of eggs developed more slowly and needed to be incubated for up to 51 days. Any eggs remaining after this time never hatched.

In natural daylight 6% of the clutches were infertile and of the fertile clutches approximately 90% of the eggs hatched successfully. Eggs produced under long- or short-day regimes became increasingly infertile,

but by the end of the experiment this reduction in fertility was less acute than that experienced by D. reticulatum.

In both species hatching of the entire clutch was completed within three days.

v) Abnormal development of A.hortensis

Wild A.hortensis are heavily pigmented, having a dark grey mantle with a black stripe running along both flanks (Fig. 310a). The foot and the alarm mucus, produced by the pedal gland, are orange/yellow. After several months in captivity some animals appear paler, the mantle becoming light grey or brown (Fig. 310b). Many of the first generation offspring had this lighter colouration from birth, but in all other respects these animals seemed normal. The second generation of hatchlings, however, appeared to lack body pigmentation, their orange/yellow colour arising from the mucus secretions alone (Fig. 310c). They soon showed other signs of abnormality which resulted in an inability to reproduce.

Newly-hatched animals had an apparently normal A-stage gonad. In contrast to their parents, however, differentiation of the spermatogonia did not begin until the slugs were at least four weeks old (Table 8).

Table 8 Abnormal development of A. hortensis

1st GENERATION

AGE	STAGE							TOTAL
	A	B	C	D	E	F/G	H	
1 DAY	3							3
1 WEEK	2	4						6
2 WEEKS		7						7
4 WEEKS		3	5					8
6 WEEKS		2	5	1				8
8 WEEKS			1	5	3			9
12 WEEKS				2	4	2		8
18 WEEKS					1	1	4	6

2nd GENERATION

AGE	STAGE							TOTAL
	A	B	C	D	E	F/G	H	
1 DAY	2							2
1 WEEK	3							3
2 WEEKS	5							5
4 WEEKS	3	1						4
6 WEEKS	2	3						5
8 WEEKS	2	3						5
12 WEEKS	1	4						5
18 WEEKS		4						4
26 WEEKS		3						3

Spermatogenesis was then halted and at 26 weeks the acini were still packed with spermatogonia and occasional, large Sertoli cells. Oogenesis was apparently unaffected and many large vitellogenic oocytes were found in the follicle cells lining the acinar wall (Fig. 311).

Development of the reproductive tract was similarly retarded, and morphogenesis was not complete until the animals were at least four weeks old. This level of development is characteristic of normal B stage animals. There was no subsequent maturation and it appears that tract development had been arrested at this stage (Figs 311 - 313).

Somatic growth continued normally and apart from their incapacity to breed, the behaviour of these animals was not affected.

Discussion

These results suggest that photoperiod is an important factor in regulating the reproductive activity of D.reticulatum and A.hortensis. The two species responded differently to extremes in daylength, reflecting their natural breeding habits in the wild.

D.reticulatum breeds throughout the year, although reproductive activity is most common during the spring and autumn months. Growth and maturation are enhanced by long-day conditions whilst short days suppress development. Since the spring generation experience increasing daylengths, growth is rapid, and the animals are sexually mature within five months. In contrast, the autumn generation develop more slowly, over a period of seven months, and their energy demands are minimal during the harsh conditions of winter. As spring approaches and the daylength increases there is a rapid burst of growth. Consequently, these animals are capable of full reproductive activity when the weather improves and their offspring have a greater chance of survival. These results demonstrate clearly that the age of an animal is no indication of the stage of reproductive development.

If copulation and oviposition commence as soon as the slugs reach maturity, a specific environmental stimulus for these activities is unnecessary. However, these experiments show that egg-production is influenced by the photoperiod. Although continuous conditions steadily reduced activity, the initial response to long daylengths was one of increased production, whilst short days significantly depressed activity. Both the frequency of egg-laying and the clutch size were affected.

A.hortensis has an annual breeding cycle and reproductive activity is intense during autumn and early winter. As in D.reticulatum growth and maturation are enhanced by long daylengths, whilst short days suppress development. Consequently, newly-hatched animals grow slowly throughout the winter months when food may be less readily available. As spring approaches and the daylength increases, the rate of development accelerates, and the animals are sexually mature within eight months.

Egg-production is again influenced by the photoperiod, but in A.hortensis short daylengths favour egg-laying whilst long days tend to have an inhibitory effect. In this species eggs are normally laid during the autumn and winter. Although the offspring of summer clutches would develop more rapidly, they would be sexually mature when conditions were least favourable for reproduction and development of the young. It is therefore advantageous if egg-laying is postponed. The development of winter hatchlings is inhibited by short daylengths and the young slugs then capitalize on the optimal growth conditions of spring and summer.

The photoperiod has a greater influence on the reproductive activity of D.reticulatum than of A.hortensis. This may reflect the importance of the timing of reproduction in the two species. Although

A.hortensis appears to have a more rigid breeding cycle, with very distinct annual peaks of activity, D.reticulatum may require greater coordination to ensure that the majority of young slugs from both generations meet the adverse weather conditions, of either summer drought or winter frost, at a stage when they are most able to survive them.

One rather obvious discrepancy arises from these results. Long days favoured egg-production in D.reticulatum while short days had a similar effect on A.hortensis, but neither of the two species laid their eggs at times in the year which corresponded to the experimental light regimes. In both cases the critical daylength probably corresponds to spring and autumn conditions and the extremes experienced had therefore passed the threshold, resulting in enhanced egg-production.

It is not surprising that the photoperiod has no effect on the length of incubation. Eggs are deposited in sheltered spots and even if the embryos could detect changes in the light conditions, they would rarely experience them.

In both species continuous conditions led to increasing infertility, especially when the animals were kept under a light regime which suppressed egg-laying.

This would not normally occur in the wild and is probably an effect of the artificial environment. In natural daylight over 90% of the clutches hatched successfully, but in the laboratory the eggs are not subjected to predators or physical stress. To guarantee that some of the offspring survive the rigours of the natural environment a high percentage of eggs must be capable of normal development. This suggests that the conditions in the animal house closely approximate conditions in the field. The seasonal fluctuations in reproductive activity also correspond closely to observations made on the wild populations, and it would appear that the controlled environment of the animal house is ideal for maintaining breeding stocks.

EXPERIMENTS TO STIMULATE EGG-LAYING

i) The effect of changing the photoperiod

It has been shown that whilst long daylengths increase egg-production in D.reticulatum, short days have the same effect on egg-laying in A.hortensis. Constant exposure to these conditions, however, does not maintain high levels of activity, and within six months production starts to decline. Changes in the photoperiod appear to be necessary for stimulating oviposition, and possibly, by transferring animals from unfavourable light regimes to ones which promote egg-laying, oviposition could be induced experimentally.

Experiment I

Materials and methods

Short daylengths suppress egg-laying in D. reticulatum. Forty animals were kept on short days (8L:16D) for two months, so that egg-production remained low. Half the group was then transferred to a long day cycle (16L:8D) and was observed at hourly intervals for the next eight hours and then regularly for a further 9 months.

The number of eggs laid by each group was recorded.

Results

Increasing the photoperiod did not stimulate egg-laying immediately and no eggs had been laid after 24 hours.

The group of animals kept on short days laid few eggs between November and July. Egg production rose slightly during April, reflecting the natural rhythm of activity, but apart from this brief increase, egg-laying continued at a consistantly low level (Fig. 314a).

Within two months the group transferred to long daylengths began to produce more eggs. There was a steady rise in production throughout February and March but then the rate of laying remained fairly constant. The average clutch size also increased with long-day conditions. By the end of the experiment egg-production had increased by 45%.

Experiment II

Materials and methods

Long daylengths tend to suppress egg-laying in A.hortensis. Thirty-six animals were kept on long days (16L:8D) for two months, and egg production fell steadily. Half the group was then transferred to a short day cycle (8L:16D) and was observed at hourly intervals for the next eight hours and then regularly for a further 8 months.

The number of eggs laid by each group was recorded.

Results

Decreasing the photoperiod did not stimulate egg-laying immediately and after 24 hours only one clutch of 25 eggs had been produced.

The group of animals kept on long daylengths laid few eggs between February and July, but production began to rise in August, reflecting the natural autumnal surge in egg-laying activity (Fig. 314b).

The number of eggs laid by the group transferred to short days did not fall to such low levels and after two months production began to increase. The rise in ovipository activity was very gradual, continuing throughout April and May. Production then remained constant and there was no further increase in activity during the autumn. By the end of the experiment egg-production had increased by 33%.

The average clutch-size increased slightly under short day conditions.

Experiment III

Materials and methods

Under natural daylight conditions egg-production in D.reticulatum starts to fall in November and does not normally increase again until March.

Forty animals were collected from the wild in mid-September and were immediately placed in the animal house. When egg-laying began to decline in late November half the group was transferred to long daylengths (16L:8D) and observed at hourly intervals for the next eight hours and then regularly for a further 9 months.

The number of eggs laid by each group was recorded.

Results

Increasing the photoperiod did not stimulate egg-laying immediately and after 24 hours only one clutch of 22 eggs had been produced.

In natural daylight egg-production continued to fall for a further two months and remained low until late February. Egg-laying then increased rapidly, reaching a peak in April, but by early summer production had started to decline once more, and fell steadily throughout the rest of the experiment (Fig. 314c).

The group transferred to long daylengths responded rapidly to their new photoperiod and the drop in egg-laying activity was halted. Production then rose steadily between January and May, and only began to decrease again in mid-April. Although this artificially induced burst of activity avoided normal winter inhibition, the number of eggs produced by both groups of animals was approximately the same, and by the end of the experiment the long-day group had only a 6% advantage.

The average clutch-size was unaffected by changing the photoperiod.

Experiment IV

Materials and methods

Under natural daylight conditions egg-production in A.hortensis is minimal between January and June.

Thirty-six animals were collected from the wild throughout November and December, and were immediately put into the animal house. Egg-production decreased in early February and half the group was transferred to a short day regime (8L:16D) and observed at hourly intervals for the next eight hours and then regularly for a further 8 months.

The number of eggs laid by each group was recorded.

Results

Decreasing the photoperiod did not stimulate egg-laying immediately and no eggs had been laid after 24 hours.

In natural daylight egg-laying remained at a low level until late July, but then increased rapidly during the autumn (Fig. 314d).

The group transferred to a short day regime responded rapidly to the decreased photoperiod and production rose steadily for the next six months, before levelling off in August and September. Although these short day animals did not show the high level of activity in autumn, their enhanced production rate throughout the spring and summer resulted in an overall increase of 22%.

The average clutch size was unaffected by changing the photoperiod.

Conclusion

In each case egg-production rose to the level expected under the new light regime (c.f. Fig. 308), but the response is delayed, and therefore unsuitable for experimental purposes. This supplies further evidence that the photoperiod affects gametogenesis and oocyte maturation rather than ovulation or egg-laying.

ii) The effect of changing the humidity

Humidity is important in regulating slug activity. Water is freely lost by evaporation and in the mucus necessary for locomotion, and as the percentage of body-water falls, all activities cease (Runham and Hunter, 1970). In addition, slugs will only lay their eggs when the soil conditions are suitable, since newly-laid eggs are particularly vulnerable to both desiccation and saturation (Carrick, 1942; Arias and Crowell, 1963).

Experiment I

Materials and methods

Adult D.reticulatum and A.hortensis were collected from the wild in mid-September, at the height of their egg-laying season. Both species were divided into four groups each containing ten animals.

Group A was placed in a sandwich box and fed on lettuce, Tetramin fish food and muesli base. The lettuce was dried to remove all surface moisture. There was no wadding to provide a humid atmosphere or shelter.

Group B animals were isolated in individual petri dishes. This prevented the animals clustering together to reduce their water loss. They experienced the same conditions as group A.

Group C was placed in a sandwich box, kept damp with moistened cellulose wadding. The animals were fed on lettuce, Tetramin fish and muesli base.

Group D animals were isolated in individual petri dishes and experienced the same conditions as group C.

Groups C and D were controls.

Each group of animals was observed daily and the incidence of egg-laying was recorded. Fresh food and wadding, where necessary, were provided every other day.

After six days all four groups were transferred to sandwich boxes and provided with moistened wadding and food. They were observed at regular intervals for eight hours and then daily for a further seven days.

Results (see Table 9)

At first the animals placed into a moisture-free environment were hyper-active, but within two hours all locomotory activity ceased.

The animals in group A started to cluster together after 30 minutes. This behaviour reduces water loss and after six days only two D.reticulatum and one A. hortensis had died of dehydration. A single clutch of 22 eggs was produced by A.hortensis on the third day but no eggs were laid by D.reticulatum.

The isolated animals were more severely affected by low humidity and the mortality was twice as high as in group A. No eggs were laid during the first six days.

Both groups sought shelter beneath the food but feeding was inhibited throughout the experiment.

When the dehydrated animals were transferred to a humid environment they remained inactive. Over the next seven days they did not resume feeding and no eggs were laid. By the end of the experiment half the animals in group A were still alive, but in group B only one D.reticulatum and three A.hortensis remained. These surviving slugs were moribund and died within one week.

Table 9 The effect of changing the humidity

Experiment I

Day		1	2	3	4	5	6	Total	Deaths	7	8	9	10	11	12	13	14	Total	Deaths
D. reticulatum																			
Group A	no. of batches	-	-	-	-	-	-	0	2	-	-	-	-	-	-	-	-	0	3
	no. of eggs	-	-	-	-	-	-	0		-	-	-	-	-	-	-	-	0	
Group B	no. of batches	-	-	-	-	-	-	0	4	-	-	-	-	-	-	-	-	0	5
	no. of eggs	-	-	-	-	-	-	0		-	-	-	-	-	-	-	-	0	
Group C	no of batches	1	-	3	1	-	-	5	0	1	-	1	-	-	-	-	1	3	0
	no of eggs	21	-	55	24	-	-	100		26	-	18	-	-	-	-	22	66	
Group D	no of batches	-	2	1	-	1	-	4	0	-	-	-	2	-	1	-	-	3	1
	no of eggs	-	39	28	-	17	-	84		-	-	-	36	-	22	-	-	58	
A. hortensis																			
Group A	no of batches	-	-	1	-	-	-	1	1	-	-	-	-	-	-	-	-	0	3
	no of eggs	-	-	22	-	-	-	22		-	-	-	-	-	-	-	-	0	
Group B	no of batches	-	-	-	-	-	-	0	3	-	-	-	-	-	-	-	-	0	4
	no of eggs	-	-	-	-	-	-	0		-	-	-	-	-	-	-	-	0	
Group C	no of batches	-	2	1	-	-	-	3	0	-	-	-	-	1	-	-	-	1	0
	no of eggs	-	58	28	-	-	-	86		-	-	-	-	27	-	-	-	27	
Group D	no of batches	-	-	-	2	1	-	3	0	1	-	-	-	-	-	-	1	2	0
	no of eggs	-	-	-	63	29	-	92		19	-	-	-	-	-	-	32	51	

The control groups, C and D, continued to produce eggs throughout the experiment.

When the isolated slugs were brought together on the seventh day, several began to show signs of early courtship behaviour and two pairs of D.reticulatum and one of A.hortensis copulated successfully. Mating activity was complete within 3.5 hours.

Conclusions

The experimental dehydration was too severe and too prolonged for normal survival, and shorter periods of water deprivation may be sufficient to suppress egg-laying without injuring the slugs.

Although this experiment failed to stimulate oviposition it did succeed in stimulating courtship behaviour and copulation in some of the control animals. Isolation appears to increase their willingness to mate and this response could be used to induce mating experimentally.

Initially D.reticulatum and A.hortensis responded to dry conditions by crawling rapidly around their new environment. In the wild this behaviour increases their chances of escaping from areas of low humidity to more favourable surroundings, but water is continually lost

by evaporation and in the mucus produced during locomotion. At some point the need to conserve body water overrides the flight response and all activities cease. The animals then seek moist shelter until conditions improve. If the water losses become too severe high humidities cannot revive the moribund slugs and death is inevitable.

Experiment II

Materials and methods

Adult D.reticulatum and A.hortensis were collected from the wild during late September. Both species were divided into five groups, each containing five animals.

Four groups were placed into sandwich boxes without wadding, while the fifth group, which acted as a control, remained in a moist environment.

The boxes were inspected daily for eggs. Fresh food, and wadding for the controls, were provided every other day.

The experimental groups were transferred to normal conditions of high humidity after one, two, three and four days. They were observed at regular intervals for eight hours and then daily for a further seven days.

Results (see Table 10)

As before the slugs became very active when first placed into a dry environment but within two hours they clustered together and were unresponsive to any mechanical stimulation.

The groups kept without moisture for only one day resumed locomotory activity within 30 minutes of increasing the humidity. During the next eight hours D.reticulatum produced a single clutch of eggs, but none of the A.hortensis was stimulated to lay. Both species laid normally throughout the following week.

The slugs which had experienced two days of dehydration also resumed normal activites within 30 minutes of raising the moisture level. After two hours two of the D.reticulatum had started to lay and an individual A.hortensis commenced laying one hour later. All ovipository activity was completed within 45 minutes. Egg-laying continued throughout the following week.

After three days dehydration the animals were initially unresponsive to increased humidities. D. reticulatum only started to move after four hours, while A.hortensis remained inactive for six hours. Both species produced a single clutch of eggs five days later.

Table 10 The effect of changing the humidity

Experiment II

Species	Duration of dehydration		Increased humidity	1	2	3	4	5	6	7	Total	Deaths
D.reticulatum	1 DAY	No. of batches	1		1				1	1	4	0
		No. of eggs	20		17				23	24	84	
	2 DAYS	No. of batches	2			1	1				4	0
		No. of eggs	47			21	21				89	
	3 DAYS	No. of batches	0					1			1	0
		No. of eggs						19			19	
	4 DAYS	No. of batches	0								0	4
		No. of eggs										
	Control (0 Days)	No. of batches	-	1			1			2	4	0
		No. of eggs		24			21			46	91	
A. hortensis	1 DAY	No. of batches	0	1				2			3	0
		No. of eggs		25				58			83	
	2 DAYS	No. of batches	1		1				1		3	0
		No. of eggs	27		29				31		87	
	3 DAYS	No. of batches	0					1			1	0
		No. of eggs						27			27	
	4 DAYS	No. of batches	0								0	5
		No. of eggs										
	Control (0 Days)	No. of batches	-		1		1	1		1	4	0
		No. of eggs			31		24	30		29	114	

Four days with no surface moisture had a debilitating affect on all the animals. Raising the humidity did not stimulate activity and the slugs remained moribund, dying within ten days. A single D.reticulatum survived and resumed egg-laying three weeks later.

The control groups laid normally throughout the experiment.

Conclusions

After two days exposure to low humidity, return to humid conditions appears to stimulate egg-laying in D.reticulatum and A.hortensis within two and 3 hours respectively. Shorter periods do not stress the animals sufficiently for them to respond positively to a more favourable environment, while prolonged dehydration is injurious to the slugs.

Experiment III

Materials and methods

Adult slugs were collected from the wild in early October. Twenty-five D.reticulatum and twenty A. hortensis were placed in sandwich boxes and kept in a moisture-free environment for two days. They were then transferred to boxes containing damp cellulose wadding and four animals from each group were injected with Susa fixative after one, two and three hours. The groups were observed for a total of eight hours and any egg-laying animals were immediately fixed for light microscopy.

Control animals were placed into a normal humid environment and their rate of egg-laying was recorded.

Results

When the reproductive tracts from the animals sampled at hourly intervals were observed histologically, none showed signs of ovulation or early egg-formation.

Only one D.reticulatum was observed laying after 2.5 hours. This animal was fixed whilst in the process

of depositing a third egg. The histological appearance of the reproductive tract will be described later.

No A.hortensis were stimulated to lay.

Conclusions

The limited success in stimulating egg-laying which was seen in experiment II was not repeated in this experiment. This suggests that although reduced humidity will suppress egg-laying, together with all other activities, increased moisture levels will not then induce oviposition with any degree of reproducibility. For this reason no further attempts were made to stimulate egg-laying by changing the humidity.

iii) The affect of changing the temperature

Slugs are normally active between 4°and 25°C, but newly-laid eggs are very vulnerable to cold and heat and ovipository activity ceases when the temperature falls

below 10° or rises above 20°C (Carrick, 1942; Barnes and Weil, 1945; Arias and Crowell, 1963).

Experiment I

Materials and methods

Adult D.reticulatum and A.hortensis were collected from the wild in mid-September. Both species were divided into three groups, each containing ten animals, which were kept at 4°, 15° and 25°C for two weeks. The low and high temperature groups were then transferred to 15°C. They were observed at regular intervals for eight hours and then daily for a further seven days.

The animals kept at 15°C acted as controls.

Results

The animals kept at 4°C immediately burrowed beneath the cellulose wadding and within two hours all activity had ceased. Feeding was minimal throughout the two week period and no eggs were laid. When transferred

to 15°C the slugs became increasingly active and fed voraciously. No eggs were produced until several days later.

The animals at 25°C also burrowed beneath the wadding and locomotory activity ceased within one hour. No food was consumed and no eggs were laid during the experiment. After one week many slugs became moribund and the groups were transferred immediately to 15°C. Only two D.reticulatum and one A.hortensis survived, but these animals had still not resumed egg-laying four weeks later.

The control groups produced eggs regularly throughout the experiment.

Conclusions

Although the extreme temperatures successfully inhibited egg-laying, this inhibition continued after conditions had improved. At 4°C feeding was also suppressed and the animals were gradually starved over the two week period.

Few slugs survived prolonged exposure to 25°C and in these animals gametogenesis may have been affected by the high temperatures, which could account for the total absence of egg-laying four weeks later. Unfortunately, the gonads of these slugs were not studied histologically.

Experiment II

Materials and methods

Adult D.reticulatum and A.hortensis were collected from the wild in late September. Both species were divided into two groups, each containing ten animals. One group was kept at 8°C for two weeks before being transferred to 15°C. The animals were observed at regular intervals for eight hours and then daily for a further seven days.

The second group acted as a control and remained at 15°C throughout the experiment.

Results

At 8°C the slugs remained active and continued feeding. However, reproductive activity was suppressed and no eggs were laid during the two week period (Table 11).

When transferred to 15°C there was no immediate change in behaviour. No eggs were laid during the first eight hours, but one day later D.reticulatum had produced three large clutches of eggs, and A.hortensis two. Egg-laying continued at an enhanced rate and after seven days nine separate egg batches had been deposited by D.reticulatum and eight by A.hortensis.

The control groups produced eggs regularly throughout the experiment.

Conclusions

Since feeding continues at 8°C the problems of starvation, encountered in the first experiment, were now overcome.

Table 11. The effect of changing the temperature

Experiment II.

	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18	19 20	21 22	Total	Average Clutch size
D.reticulatum													
8°C No. of clutches								3	2 2	1	1	9	27
No. of eggs								88	53 50	29	26	246	
15°C No. of clutches	1		2			1			1	1		6	22
No. of eggs	21					18			23	22		129	
A. hortensis													
8°C No. of clutches								2	1 1	2 1	1	8	33
No. of eggs								71	34 31	59 37	28	260	
15°C No. of clutches		1		1 1	1		1	1		1		7	29
No. of eggs		27		29 31	30		26	29		30		202	

Ovipository activity was successfully inhibited in both species and higher temperatures then encouraged egg-laying. Assuming that each animal deposits only one clutch of eggs within a seven day period, nine out of ten D.reticulatum and eight out of ten A.hortensis had been stimulated to lay. In addition, the egg-batches tended to be larger, D.reticulatum producing 24-32 eggs at a time, whilst A.hortensis produced clutches of up to 38 eggs. Under normal conditions the average clutch size is 22 and 29 eggs respectively.

Unfortunately, this response to more favourable conditions was delayed and the first eggs were deposited 24 hours after the initial stimulus. Consequently, this technique for stimulating egg-laying is not suitable for investigating ovulation and egg-formation prior to oviposition.

iv) The effect of cleaning and feeding

The laboratory stock animals frequently laid eggs a few hours after they had been cleaned and provided with fresh food. Earlier experiments have shown that low temperatures ($4^{o}C$) suppress feeding. As soon as this inhibition is removed the animals eat voraciously and do not resume normal reproductive activity for several days. Stress due to starvation, therefore, is

not suitable for stimulating oviposition experimentally. Deterioration of the environment, however, may suppress egg-laying until conditions improve.

Materials and methods

Adult D.reticulatum and A.hortensis were taken from the stock supply during October. Both species were divided into two groups, each containing ten animals.

One group was given a large supply of food and left for seven days. During this time the carbon dioxide levels rose and conditions within the sandwich boxes deteriorated. The animals were then transferred to clean boxes, without food and observed regularly for eight hours.

The second group of animals acted as controls and were cleaned and fed every other day as usual. Each box was observed at regular intervals after cleaning.

Results

After seven days in an increasingly foetid environment D.reticulatum and A.hortensis had produced a total of 28 and 61 eggs respectively. Excess food still remained.

When the animals were provided with fresh air and clean surroundings they became very active and within one hour a single A.hortensis had commenced egg-laying. This animal was immediately fixed for light microscopy and the histological findings will be discussed later. After eight hours no further eggs had been deposited. One animal died during the experiment.

The control groups laid eggs regularly throughout the experiment. However, no eggs were deposited immediately after cleaning and no animals were caught in the act of oviposition.

Conclusions

A foetid environment probably suppresses egg-laying. Since the boxes were left unopened during the experiment it was impossible to observe the rate of oviposition, but it is likely that the eggs produced by both species had been laid within the first few days. Fresh conditions, therefore, may be important for ovipository activity but the stimulatory effect is not proven.

In the wild animals are likely to crawl away from similar polluted conditions.

v) Injection of homogenates of cerebral ganglia

Homogenates of tissues, thought to contain ovulation or egg-laying hormones, have been injected into a few species of molluscs in an attempt to stimulate oviposition. In Aplysia californica both ovulation and egg-laying can be induced by injecting homogenates of bag cells, neurosecretory cells in the abdominal ganglion, into reproductively mature animals (Kupfermann, 1967, 1970). Similarly, homogenates of cerebral commissures, containing the caudo-dorsal cells, will stimulate egg-laying in Lymnaea stagnalis (Geraerts and Bohlken, 1976). In both species there are seasonal fluctuations in their response.

Takeda (1977) reported that D.reticulatum and Limax flavus could be stimulated to lay eggs by injecting homogenates of their cerebral ganglia. The response was enhanced by removing the optic tentacles. Takeda's procedures for preparing and injecting extracts were copied in an attempt to stimulate egg-laying in both D.reticulatum and A.hortensis.

Experiment I

Materials and Methods

Cerebral ganglia were removed from mature D.reticulatum and A.hortensis and immediately homogenized in cold, 0.9% physiological saline (Hedon-Fleig; 0.1 ml saline/cerebral ganglion). The homogenates were kept on ice.

Twenty D.reticulatum and twenty A hortensis were anaesthetized using the technique designed by Bailey (1969) and the tentacles were removed from ten animals in each group. The homogenates were injected into the body cavities of five intact and five tentaclectomized animals, at a dose of four cerebral ganglia per slug (0.4 ml). The remaining animals acted as controls and were injected with the same volume of cold saline. Each slug was isolated and observed closely for signs of egg-laying.

These experiments were performed in September when both species are at their peak of egg-laying.

The size of the experiment was limited by the large numbers of animals required for each injection.

Results

The results are shown in Table 12 . No eggs were laid in the first 24 hours. Eggs were produced by at least one member of each group within seven days of injection, but production was not enhanced by injection of the cerebral ganglia or by removal of the tentacles.

Experiment II

The animals may have laid shortly before the start of the experiment and their response would be restricted by their ability to lay eggs. The experiment was repeated using animals which had not produced eggs for at least seven days.

Results

The results are shown in Table 12. As in the previous experiment there was no enhanced response by animals injected with cerebral ganglia or minus their tentacles.

Table 12 Injection of homogenates of cerebral ganglia - The number of eggs laid within 7 days of injection

Experiment I

	Materials injected	State of animal	1	2	3	4	5
D. reticulatum	Brain homogenates	Intact	0	0	17	0	22
		tentacles removed	0	0	2	died	18
	Physiological saline	Intact	23	0	0	0	25
		tentacles removed	0	0	0	20	0
A. hortensis	Brain homogenates	Intact	0	21	0	0	0
		tentacles removed	0	0	28	17	0
	Physiological saline	Intact	0	0	9	0	0
		tentacles removed	0	23	0	0	0

Experiment II

	Materials injected	State of animal	1	2	3	4
D. reticulatum	Brain homogenates	Intact	died	0	0	0
		tentacles removed	22	0	0	0
	Physiological saline	Intact	0	0	15	0
		tentacles removed	0	0	23	0
A. hortensis	Brain homogenates	Intact	0	0	0	12
		tentacles removed	0	29	0	0
	Physiological saline	Intact	0	died	died	19
		tentacles removed	0	0	6	0

Conclusions

Takeda's experiments could not be reproduced. It has since been revealed that his preparation of the homogenates involved repeated freeze-thawing of the tissue to release the active hormone (Runham, pers. comm.).

Time and the availability of animals prevented further work on this aspect of egg-laying.

Summary

By altering the environment egg-laying can be suppressed or enhanced experimentally in both D. reticulatum and A.hortensis. Unfavourable photoperiods, low humidity, freezing temperatures, starvation and foetid conditions will all suppress oviposition. Improving the environment will frequently stimulate egg-laying in reproductively-active animals, but the timing of this response varies from a few hours to several days. To study the events which lead to the production of a fertilized egg it is necessary to predict ovulation with a high degree of success. The results of these experiments do not guarantee such success and other stimuli must be considered.

Lovett and Black (1920) observed that D. reticulatum burrowed into the soil and became quiescent 1-3 days before egg-laying. If this were a reproducible feature of slug reproductive behaviour it could be used experimentally to indicate a slug's readiness to ovulate and lay eggs. Throughout this investigation however there were no observable changes in behaviour prior to egg-laying. Animals maintained in the laboratory normally remain quiescent for long periods, only becoming active to eat and occasionally reproduce. In these animals, therefore, quiescence is no indication of imminent ovipository activity.

EXPERIMENTS TO STIMULATE COPULATION

Previous experiments (Page 200) suggested that isolation stimulates mating in both D.reticulatum and A.hortensis.

This technique has been used to induce copulation in Helix pomatia (Lind, 1968) and amongst members of the genus Partula (Lipton and Murray, 1979). In these animals it is necessary to isolate the snails for a minimum of one week, and longer periods appear to enhance the response.

Materials and methods

Adult D.reticulatum and A.hortensis were collected from the wild and isolated in petri dishes. In all other respects conditions were normal.

After one, two and three weeks ten animals of each species were grouped together and observed for signs of courtship behaviour and copulation. They were illuminated by red light.

Results (See Table 13)

D.reticulatum

After one week of isolation four animals responded to the other members of the group by protruding their sarcobella. This is the initial stage of courtship and was first seen thirty minutes after combining the animals. The last to respond commenced courtship one hour later. These stimulated slugs paired up, and after courtships lasting 25 and 105 minutes they copulated successfully and then separated.

After two weeks isolation five animals became sexually responsive within two hours of being grouped together. Two pairs mated successfully after courtships lasting 55 and 85 minutes. The fifth animal, however,

Table 13a Experiments to stimulate copulation

D. reticulatum

Period of isolation	Time before everting sarcobellum (minutes)	Pairs	Duration of courtship (minutes)	Successful copulation	Total no. stimulated	Total no. unaffected
1 WEEK	1. 30 2. 45	]	25	y	4	6
	3. 65 4. 90	]	105	y		
2 WEEKS	1. 35 2. 45	]	55	y	5	5
	3. 85 4. 95	]	85	y		
	5. 120		25	N		
3 WEEKS	1. 10 2. 15	]	40	y	3	7
	3. 20		15	N		

Table 13 b Experiments to stimulate copulation

A. hortensis

Period of isolation	Time before pairing (minutes)	Pairs	Duration of courtship (minutes)	Successful copulation	Total no. stimulated	Total no. unaffected
1 WEEK	1.] 35 2.]	]	125	N	2	8
2 WEEKS	1.] 45 2.]	]	130	y	4	6
	3.] 60 4.]	]	155	N		
3 WEEKS	1.] 30 2.]	]	180	y	4	6
	3.] 165 4.]	]	115	N		

found no partner and withdrew its sarcobellum within half an hour.

Three weeks of isolation produced a more rapid response. The first animal everted its sarcobellum after only ten minutes and its partner followed just five minutes later. Copulation was complete within three quarters of an hour. A third animal was also stimulated to mate but it failed to pair up and became unresponsive after 15 minutes.

A.hortensis

The first indication that A.hortensis are ready to mate is when they form pairs. The individual response cannot be assessed earlier since there is no external show of sexual excitability.

After one week in isolation two slugs paired after 35 minutes. Courtship lasted just over two hours but the animals separated before the sperm packages had been exchanged.

In the group which had experienced two weeks of isolation two pairs commenced courtship within one hour. After a further two hours one of these pairs copulated successfully, but the other separated without exchanging sperm.

Three weeks isolation also stimulated courtship behaviour in at least four animals. The first couple paired up after thirty minutes and copulation was completed within 2.5 hours. The second pair responded more slowly and courtship was only initiated after 2.75 hours. These animals failed to copulate and separated approximately two hours later.

Conclusions

From these observations the length of isolation does not appear to be critical for stimulating reproductive activity.

D.reticulatum respond equally rapidly and with similar success to one or two weeks separation. Three weeks isolation accelerated the response but fewer animals were stimulated to mate.

The experiments with A.hortensis were slightly less effective and resulted in fewer successful matings. Isolation periods of two and three weeks produced the best response.

Consequently, two weeks of isolation were considered preferable for stimulating courtship behaviour and copulation in both D.reticulatum and A. hortensis.

Subsequent experiments showed that red light was unnecessary and the same frequency of copulation could be achieved with daylight illumination.

EXPERIMENT TO SEE IF COPULATION OR THE PRESENCE OF SPERM IN THE REPRODUCTIVE TRACT STIMULATES OVULATION AND OVIPOSITION.

In Lymnaea stagnalis the sperm received at copulation passes directly into the bursa copulatrix. Horstmann (1955) demonstrated that only a small proportion of this sperm continues on into the common duct where it is transported by ciliary action along the female groove towards the carrefour. The large mass of sperm remaining in the bursa stimulates the snail to ovulate and eggs are produced one day after mating.

In D.reticulatum and A.hortensis eggs are seldom laid immediately after copulation, and mating itself is unlikely to stimulate ovulation. However, if some stimulus resulting from copulation, e.g. the passage of sperm along the reproductive tract or its arrival at some specific site, such as the carrefour, regulates the release of ova, the eggs would appear after a constant time interval.

Materials and methods

Adult D.reticulatum and A.hortensis were collected from the wild and isolated in petri dishes as before.

After two weeks 20 D.reticulatum and 18 A.hortensis were grouped together. Copulating pairs were removed immediately after separation and returned to their individual petri dishes. The appearance of the first egg-batch was recorded for each animal.

Results (See Table 14)

Fourteen out of the 20 D.reticulatum became sexually active when grouped together and twelve pairs copulated successfully. The first eggs appeared 4 to 41 days later but after six weeks three animals had still not started to lay.

Eight out of the 18 A.hortensis formed mating pairs and every one proceeded through to copulation. The first eggs were seen 14 to 48 days later, but by six weeks two animals had still not commenced laying.

Table 14 Experiment to see if copulation or the presence of sperm in the reproductive tract stimulates ovulation and oviposition

D. reticulatum

PAIR	A		B		C		D		E		F	
ANIMAL	1	2	3	4	5	6	7	8	9	10	11	12
TIME INTERVAL, IN DAYS, BEFORE 1st EGG BATCH	41	10	21	-	4	-	-	4	19	9	36	17
SIZE OF EGG BATCH	42	19	12	-	1	-	-	21	30	5	25	16

A. hortensis

PAIR	A		B		C		D	
ANIMAL	1	2	3	4	5	6	7	8
TIME INTERVAL, IN DAYS, BEFORE 1st EGG BATCH.	48	25	35	-	23	14	-	22
SIZE OF EGG BATCH.	33	26	31	-	18	27	-	29

Conclusions

The time interval between copulation and production of the first clutch of eggs was very variable for both species, which suggests that the stimulus for ovulation is unrelated to copulation or movement of spermatozoa. If the oocytes had been released within hours of mating, they would have had to be stored for periods of up to 41 days in D.reticulatum and 48 days in A.hortensis. Any oocytes or zygotes stored in the reproductive tract for six weeks would undoubtedly have been observed in normal, routine histological sections. In fact oocytes are only seen in the reproductive tracts of animals which have been fixed while in the process of egg-laying.

Therefore, some other factor must be involved in regulating ovulation.

COPULATION AND EGG-LAYING IN D. RETICULATUM AND A. HORTENSIS

COPULATION

Adult D.reticulatum and A.hortensis were isolated for two weeks. They were stimulated to copulate as above and detailed descriptions of the events leading up to the exchange of sperm were recorded. Pairs were then sampled for histological study at timed intervals during courtship and again after copulation.

Copulation in D. reticulatum

1) Diary of the events leading up to sperm transfer in a typical mating pair

mins.:secs.

00:00 Ten adult D. reticulatum, which have been isolated for two weeks, are placed together.

20:30 First slug (A) protrudes sarcobellum and begins to circle around second slug (B).

21:15 (A) repeatedly nudges (B) with its head.

22:15 (B) protrudes sarcobellum.

22:45 The pair start to circle slowly around one another (Fig. 315a). This circling continues throughout courtship.

23:00 The pair caress each other with the tips of their sarcobella (Fig. 316a).

23:45 (A) protrudes buccal mass and strikes out towards (B)'s sarcobellum (x3).

24:30 The pair separate and circle away from each other chasing their own tails (Fig. 315b).

25:30 (A) withdraws sarcobellum.

25:45 (B) strokes (A) with tip of sarcobellum, particularly around the pulmonary opening.

26:15 (A) protrudes sarcobellum.

26:30 The pair caress each other with their sarcobella and gradually move closer together (Figs. 315c & 316b).

50:00 The genital openings are apposed. The sarcobella meet at their bases and become erect for 1 to 2 seconds. The pair

immediately separate and continue caressing (x6) (Fig. 316c).

54:00 (A) erects sarcobellum.

55:30 Genital openings apposed and sarcobella erected for 1 to 2 seconds (x5).

60:30 (A) turns away to follow its own tail. (B) continues to caress (A).

61:00 Resume previous positions.

61:15 Genital openings apposed. Sarcobella erected for 10 seconds.

62:00 Genital openings apposed. Sarcobella erected for 1 to 2 seconds.

63:15 Genital openings apposed. Sarcobella erected for 15 seconds.

63:45 Genital openings apposed. Sarcobella erected for 1 to 2 seconds (x2).

64:30 Genital openings apposed. Sarcobella erected for 10 seconds.

64:45 Genital openings apposed. Sarcobella erected for 1 to 2 seconds (x5).

69:45 Genital openings apposed. Sarcobella erected for 50 seconds. Tips of sarcobella become entwined.

71:00 Genital openings apposed. Sarcobella erected for 20 seconds.

72:00 Genital openings apposed. Sarcobella erected for 1 to 2 seconds (x4).

75:15 Genital openings apposed. Sarcobella erected for 35 seconds. Bases of sarcobella appear to pulsate.

79:45 Genital openings apposed. Sarcobella erected for 1 to 2 seconds (x5).

82:30 Genital openings apposed. Sarcobella erected for 80 seconds. Bases of sarcobella appear to pulsate. The penial sac of (A) becomes partially everted but is then withdrawn.

85:15 Genital openings apposed. Sarcobella erected for 40 seconds. Bases of sarcobella appear to pulsate. Partial eversion of both penial sacs (Fig. 316d).

86:00 (B) withdraws tentacles.

86:30 (A) withdraws tentacles.

86:45 Both put out tentacles.

87:00 Genital openings apposed. Sarcobella erected and appear rigid.

87:15 Simultaneous and complete eversion of the penial sacs (Figs. 316e — 316h). The sarcobella are displaced to one side and the penial sacs are locked together (Fig. 316e). The white, sperm masses can be seen on the surface (Fig. 316f). The everted trifid appendages lie over each partner (Figs. 316g & 316h). The pair circle rapidly, twisting the conjoined penial sacs and the sperm-masses are exchanged.

87:45 The pair separate.

87:50 The penial sacs are withdrawn.

87:55 The sarcobella are withdrawn.

Response time 20:30 minutes

Courtship 66:15 minutes

Sperm exchange 0:30 seconds

2) General description of courtship and copulation

When a slug is stimulated to mate either naturally or artificially, it first everts the sarcobellum. It may then incite a second receptive slug to commence courtship behaviour by gently nudging the animal with its head, or stroking it with the sarcobellum. If the second animal responds, the pair start to circle slowly in a clockwise direction and this continues throughout courtship (Figs. 317a - e). They caress one another with just the tips of their sarcobella for approximately 5 minutes (Fig. 316a). During this time one, or both, may protrude the buccal mass and attempt to rasp the other around the pulmonary and genital openings. Occasionally, this irritates the partner who may become unresponsive and crawl away, but more usually it further stimulates activity.

The pair then begin to caress one another with the whole length of their sarcobella and gradually move closer together (Fig. 316b). After 20 to 30 minutes of mutual stimulation the genital openings are apposed, momentarily, and the two sarcobella become erect (Figs. 316c & 317c). This can be repeated up to 30 times. Periodically, the mating position is held for 10 to 15 seconds, and the tips of the sarcobella may be entwined. As the climax of copulation approaches, this occurs more frequently, and eventually it is sustained for 1 to 2 minutes. The bases of the sarcobella now appear to pulsate and there is partial eversion of the penial sacs (Figs. 316d & 317f). This may be repeated 3 to 4 times before both animals are ready to exchange sperm.

After courtships lasting 0.5 to 1.5 hours the slugs may withdraw their optic tentacles for a few seconds. The genital openings are apposed for the last time and the sarcobella become erect. As their bases pulsate the whole structure swells and appears turgid. The penial sacs are then everted simultaneously, and become locked together, displacing the sarcobella to one side (Figs. 316e & 317g). The slugs circle rapidly, twisting the interlocked penial sacs, and transferring the white, sperm packages from one animal to the other (Figs. 316f, 317h & 317i). The everted trifid appendage appears to play no part in sperm exchange and lies

passively across the back of the partner slug (Figs. 316g & 316h).

Copulation lasts only 30 seconds. The animals then separate and immediately withdraw their penial masses.

Throughout courtship and copulation copious mucus is constantly being produced. This appears to attract other slugs who may be stimulated to protrude their sarcobella, and 30 minutes after copulation the mucus will still interest unmated individuals.

Any sperm remaining after the penial masses have withdrawn is immediately eaten, along with the accumulated mucus secretion.

3) The fate of the sperm at timed intervals during courtship

10 minutes after protrusion of the sarcobellum

The gonad appears normal and there is no evidence that courtship stimulates the immediate release of spermatozoa into the hermaphrodite duct.

For most of its length the hermaphrodite duct functions as a seminal vesicle. Within ten minutes of protruding the sarcobellum some of the stored sperm starts to flow along the slender, coiled portion of the duct and into the pouched chamber of the carrefour (Fig. 318). At this stage, however, the sperm does not fill the pouched diverticulum, but passes directly into the base of the carrefour (Fig. 319) and then into the male groove of the common duct (Fig. 320).

During this time the sub-epithelial mucocytes around the carrefour diverticula become enlarged and their secretion is released into the lumen of the horseshoe-shaped pouch (Fig. 321). The prostate gland also becomes active when courtship commences, and small amounts of secretion begin to accumulate along the male groove of the common duct.

The anterior portion of the reproductive tract is unchanged.

15 minutes after protrusion of the sarcobellum

The whole mass of stored sperm has moved anteriorly and the distal half of the hermaphrodite duct is almost empty (Fig. 322).

The sperm has now filled the pouched diverticulum of the carrefour and this has expanded to accomodate the large volume (Fig. 323). No sperm, however, has entered the finger-like diverticulum.

The flow of sperm into the common duct has halted and only small traces of sperm remain in the male groove.

By this time both the albumen and carrefour glands are secreting. The collecting ducts of the albumen gland are full of an amorphous secretion which overflows into the oviducal side of the common duct. while the sub-epithelial secretory cells of the carrefour gland release their secretion directly into the lumen of the male groove (Fig. 324).

Prostate gland activity has greatly increased. The apices of the secretory cells are pale-staining, having recently released their product into the prostate tubules (Fig. 325).

This secretion now fills the entire length of the male groove (Fig. 326), and has progressed through the vas deferens to the penial sac (Fig. 327). Here it has accumulated, filling the expanded base of the trifid appendage. The small amount of sperm which had passed directly into the common duct at the onset of courtship,

has been carried along with this prostate secretion and is now seen at the centre of the secretory mass.

20 minutes after protrusion of the sarcobellum

The carrefour is now empty and has resumed its normal dimensions (Fig. 328). The mucocytes are still prominent and continue to release their secretion into the pouched diverticulum.

The sperm has now progressed into the male groove of the common duct which expands to accomodate the large volume (Fig. 329). Just before the duct divides into the vas deferens and free oviduct, however, the volume exceeds the capacity of the male groove and some sperm overflows into the oviducal lumen. There is little prostate secretion released at this stage.

The sperm flows rapidly along the common duct and a large quantity has already passed through the vas deferens into the penial sac. The vas deferens is now fully distended and here the sperm has become arranged in discrete bundles surrounded by the prostate secretion (Fig. 330). These bundles accumulate in the penial sac near the base of the trifid appendage (Fig. 331).

The large volume of secretion which had preceded the sperm mass has now passed up into the trifid diverticula and the side branches have swollen until they appear almost spherical (Figs. 332 & 333). The epithelium becomes squamous as the ductule distends (Fig. 334).

30 minutes after protrusion of the sarcobellum

Secretion from the albumen gland continues to fill the collecting ducts and a large volume has spread into the oviducal side of the common duct.

The male groove and vas deferens are once again filled with prostate secretion (Fig. 335), but the activity of the prostate gland has been decreasing and, at this stage, very little secretion is seen within the prostate tubules.

All the sperm has now passed into the penial sac.

40 minutes after protrusion of the sarcobellum

Spermatozoa have now been released from the gonad, filling the ductules which lead into the hermaphrodite duct and replenishing the store of sperm in the seminal vesicle (Fig. 336).

The mucocytes, the albumen gland and the prostate gland are no longer releasing their copious secretions, although the carrefour gland still remains active.

The final secretion from the prostate gland has now passed into the penial mass, to complete the sperm package (Figs. 337 & 338), and the male groove and vas deferens appear empty. Excess secretion fills the blind-ending side branches of the trifid appendage, and the main diverticula now become occluded by the distended cell apices, which were previously seen in non-copulatory animals (Fig. 339).

Prostate gland secretion

Four types of secretory granule could be distinguished in the prostate gland using the light microscope, and these were characterized according to their staining properties with Weigert's iron haematoxylin, Biebrich scarlet - methyl blue variation.

Type A - small, dense, purple granules (diam. 1 - 2μm) which are scattered throughout the cytoplasm of the cell giving it a highly speckled appearance (Fig. 340). These secretory cells are most abundant in the anterior region of the common duct where they are concentrated around the male groove or at the periphery of the gland.

Type B - granules having a dense purple core (diam. 1 - 2μm) surrounded by a pale blue/purple corona (total diam. 3 - 5μm) (Fig. 341). They are most frequently seen in the central region of the common duct.

Type C - pink-staining granules (av. diam. 1.5μm) (Fig. 342) which are dispersed throughout the prostate gland.

Type D - large, pale blue granules (diam. 2 - 3μm) with vesicular contents (Fig. 343). These form the bulk of prostate secretion.

Types A, B and D contribute to the sperm package while the diverticula of the trifid appendage are filled with granule types A, C and D (Table 15).

In the sperm package the sperm and secretions are not interspersed. The sperm bundles are completely (Fig. 344) or partially (Fig. 345) surrounded by the large, pale type D granules (Fig. 346) and the entire mass is then ensheathed by the cored vesicles (type B)

and a thin, outer layer of type A granules (Figs. 346 & 347).

The side branches of the trifid appendage are mainly filled with pink granules (type C). These are surrounded by a thin layer of pale blue secretion (type D) which contains occasional clusters of the small, dense, type A granules (Fig. 348).

Table 15 Distribution of the prostate secretion granules

	Prostate gland	Sperm package	Trifid appendage
Type A	+	+	+/-
Type B	++	++	-
Type C	++	-	+++
Type D	+++	+++	++

+++ abundant; ++ common; + present in low numbers; +/- occasionally seen; - absent.

The sperm package

The fully-formed sperm package has a fat, fusiform shape (Fig. 349) which does not appear to have any surface architecture when examined in the scanning EM (Fig. 350), and in places the sperm are seen through the thin layer of secretion (Fig. 351).

4) The fate of the sperm at copulation

Copulation lasts only 30 seconds. The penial sac is everted through the genital opening and swells rapidly to approximately five times its resting size (Fig. 352). As it swells the epithelium stretches, becoming cuboidal and losing its characteristic crenellations (Fig. 353). The sub-epithelial connective-tissue and musculature also become very stretched and consequently they appear more widely dispersed. The oviduct and the bursa copulatrix now open directly to the exterior via a narrow, slit-like groove which forms between the everted penial sac and the body wall (Figs. 352 & 354).

The sperm package lies on the surface of the freshly everted penial mass, closely applied to the crenellated, thickened wall of the penial sac (Fig. 355). It is transferred rapidly to the partner, however, and

prior to withdrawal of the penial mass the anterior portion is seen within the groove leading to the bursa and oviduct (Fig. 356).

The trifid appendage is the last to evert, and in animals fixed during the first few seconds of copulation the trifid diverticula still retain their normal configuration (Figs. 357 & 358). Immediately upon eversion the trifid appendage loses all the accumulated prostatic secretion (Fig. 359).

The pressure required to evert the penial sac displaces other organs within the haemocoel, and the digestive tract is frequently found within the everted penial mass (Fig. 352). The increased pressure also affects the sarcobellum, which swells as the central sinus fills with blood (Figs. 352 & 360).

The penial masses do not establish an intimate connection while exchanging the sperm and on fixation they disengage. Consequently it is impossible to prepare conjoined specimens for light microscopy.

5) The fate of the sperm at timed intervals after copulation

10 minutes after copulation

The transferred sperm now lies within the penial sac and is closely applied to the thickened, crenellated ridge which bears the sarcobellum (Fig. 361). The anterior portion of the sperm package has entered the bursa copulatrix but only extends into the proximal, unciliated region (Fig. 362). The large, ciliated digestive saccule contains none of the newly received sperm (Fig. 361).

Both the oviduct and vas deferens appear empty. The trifid diverticula have not fully withdrawn and still remain partially everted (Fig. 363).

20 minutes after copulation

There are no significant changes in the distribution of the sperm or in the appearance of the reproductive tract.

30 minutes after copulation

The sperm has moved further into the bursa copulatrix and the narrow entrance has dilated to accomodate the sperm package (Fig. 364).

A small quantity of sperm remains near the base of the trifid appendage.

40, 50 and 60 minutes after copulation

Most of the sperm package has now entered the bursa but the entrance is still widely dilated (Figs. 365 & 366).

75 minutes after copulation

Only a small amount of sperm remains in the penial sac, at the base of the trifid appendage. The rest has passed into the bursa copulatrix (Fig. 367) and the entrance to the bursa has resumed its normal dimensions.

The trifid diverticula are now fully inverted.

90 minutes after copulation

The sperm remaining in the penial sac has been channelled towards the vas deferens and appears to be entering this slender duct (Fig. 368).

4 hours after copulation

Sperm now fills the finger-like diverticulum of the carrefour (Fig. 369) but the pouched diverticulum, the common duct and the vas deferens all remain empty.

A small quantity of sperm is still present in the penial sac.

6 hours after copulation

The sperm has now passed from the finger-like diverticulum into the expanded pouch (Fig. 370).

Sperm is still present in the penial sac.

8 hours after copulation

The sperm has progressed from the pouch to the slender portion of the hermaphrodite duct (Figs. 371 & 372). It is a very small volume and fills only 1/3 of the duct's total length.

The penial sac is now empty of sperm.

24 hours after copulation

The only evidence indicating that the animal has recently copulated is the appearance of the bursa copulatrix which is filled with a disorganized mass of sperm, prostate secretory granules and unidentifiable debris (Fig. 373).

The rest of the reproductive tract appears normal.

Copulation in A.hortensis

1) Diary of the events leading up to sperm transfer in a typical mating pair

mins.:secs.

000:00 Ten adult A.hortensis, which have been isolated for two weeks, are placed together.

33:00 Two animals pair up with their genital openings apposed (Fig. 374a). They start to circle very slowly, but their movement is barely detectable.

56:00 The pair separate slightly for 2 to 3 seconds, revealing the cream-coloured lower atrium at the genital opening (Fig. 374b).

80:45 Pair separate slightly for 2 to 3 seconds.

115:00 Pair separate slightly for 2 to 3 seconds.

139:00 Pair separate slightly for 2 to 3 seconds.

162:15 Pair separate slightly for 2 to 3 seconds.

191:45 Pair separate slightly for 2 to 3 seconds.

211:30 Pair separate slightly for 2 to 3 seconds.

213:00 Slug (A) everts the glandular, lower atrium and partially everts the upper atrium exposing the conical pad of the epiphallus (Fig. 374c).

213:05 Slug (B) imitates this behaviour and the two atria engage.

213:10 Simultaneous and complete eversion of the upper atria (Figs. 374d & 374e).

213:15 The upper atria expand and slowly caress the backs of the partner slug (Fig. 374f).

213:30 Everted organs are withdrawn.

213:35 Pair separate.

Response time	33:00 mins.
Courtship	180:00 mins.
Sperm exchange	0:30 secs.

2) General description of courtship and copulation

When A.hortensis are stimulated to mate there are no external clues to their sexual excitability. Only when two receptive animals have paired up, with their genital openings apposed, can their intent to mate be recognized (Fig. 374a).

The animals remain in this position for up to 4 hours, circling very slowly in a clockwise direction, movement is barely detectable and some pairs become stationary after 30 minutes. Approximately every half hour the slugs separate momentarily, revealing the

cream-coloured lower atria just within the dilated genital openings (Fig. 374b).

After courtships lasting 2 to 4 hours one slug will evert the lower atrium and part of the upper atrium so that the conical pad which crowns the opening of the epiphallus is now exposed (Fig. 374c). The second slug responds immediately by everting its own atria and the two animals become locked together (Fig. 375). Within five seconds the upper atria further evaginate (Figs. 374d & 374e) and as they expand two parallel flanges or ridges spread outwards along the lower surface (Fig. 374f). The thick muscular oviducts can be seen clearly through the atrial wall. The animals stroke their extended upper atria along the backs of their partners.

Copulation lasts only 30 seconds. The atria are then withdrawn and the pair separate immediately.

If copulation is asynchronous, i.e. the upper atria are everted at slightly different times, the wasted spermatophores are eaten before the slugs move away.

A.hortensis were never observed copulating in the wild.

3) The fate of the sperm at timed intervals during courtship

30 minutes after the start of courtship

No sperm has been released from the hermaphrodite duct and the reproductive tract appears normal.

60 minutes after the start of courtship

The activity of the carrefour gland has increased and its secretion fills the male groove at the start of the common duct (Fig. 376). Still no sperm has been released from the hermaphrodite duct.

90 minutes after the start of courtship

An aliquot of sperm has now been released from the seminal vesicle. In some animals it still fills the slender, coiled portion of the hermaphrodite duct (Fig. 377), but in others all the sperm has passed into the descending limb of the carrefour (Fig. 378). Here the sperm accumulates and is easily accomodated without expansion of the duct. A small amount of sperm may flow through into the proximal region of the ascending limb, but it never extends beyond the start of the carrefour gland (Fig. 378).

Both the carrefour and albumen glands are releasing secretion.

120 minutes after the start of courtship

The sperm has now passed from the carrefour to the male groove of the common duct (Fig. 379). At the same time the prostate gland has become very active and the prostate tubules are filled with secretion (Fig. 380). Many of the secretory cells have already released their contents and now appear empty (Fig. 381). The secretion pours into the male groove and mingles with the sperm as it flows anteriorly (Fig. 382). The duct expands to accomodate the increased volume (Fig. 379).

After 2 hours the combined mass of sperm and secretion has reached the anterior end of the common duct and is just entering the vas deferens (Fig. 379).

Prostate gland secretion

Only two of the granule types seen in the prostate gland of D.reticulatum could be identified in A.hortensis. Type B secretory granules were concentrated in the posterior half of the common duct, while type D granules were present throughout the gland and formed the major component of prostate secretion.

The spermatophore

The fully-formed spermatophore has a lanceolate shape with a single longitudinal ridge of curved spines (Fig. 383). The central mass of sperm is mixed with secretion from the prostate gland (granule types B and D). At the anterior, head, end the outer surface of the sperm mass is surrounded by concentric layers of the pale blue, type D secretion, containing scattered type B granules (Fig. 384). Towards the periphery the cored granules diminish and the secretion forms an homogeneous sheath. As these outer layers harden they assume a pink colour which darkens into a deep red. Posteriorly, this outer red coat is much thinner and the lamellae are absent (Fig. 383).

4) The fate of the sperm at copulation

Copulation lasts only 30 seconds. Both atria are everted through the genital opening (Fig. 385), but whereas the glandular lower atrium has little capacity for expansion (x 1.1) the muscular upper atrium swells to approximately three times its natural size (Fig. 386). As the atria dilate the epithelium becomes very stretched so that the tall columnar epithelial cells now appear cuboidal or even squamous (Fig. 387). Consequently, the sub-epithelial secretory cells of the lower atrium and the thick layer of obliquely orientated

muscle surrounding the upper atrium, become very widely dispersed (Figs. 388 & 389).

The anterior genital ducts are now open to the exterior. The glandular cone which surrounds the entrance to the epiphallus protrudes from the surface of the upper atrium as a distinctive knob or button (Fig. 385). The bursa copulatrix, however, does not open directly onto the newly exposed atrial surface. Part of the upper atrium surrounding the entrance to the bursa does not fully evert, but forms a channel which links the bursa to the exterior (Fig. 390). Immediately upon eversion, the cone of the epiphallus fits into the channel of the partner, temporarily uniting the two animals (Figs. 375, 391, 392).

The thick, muscular oviduct lies within the expanded upper atrium, opening out near the apex of this elongated structure (Figs. 386 & 390).

Most of the sperm has now passed from the vas deferens into the proximal region of the epiphallus where it assumes the shape of the papillated epithelium (Fig. 393). At this stage it has not received the outer coating of the completed spermatophore. The addition of this outer coat was not observed.

5) The fate of the sperm at timed intervals after copulation

Immediately after copulation

The tip of the spermatophore has entered the bursa copulatrix causing the short muscular duct to dilate (Fig. 394). Most of the spermatophore, however, lies within the upper atrium where it is bathed in a pale-staining secretion (Fig. 395). This secretion extends into the lower atrium and also along the length of the oviduct (Figs. 396 & 397).

2 hours after copulation

A small amount of sperm remains within the upper atrium (Fig. 398). The main bulk of the spermatophore now lies within the bursa copulatrix, and the duct has resumed its normal dimensions. No sperm is seen elsewhere in the anterior portion of the reproductive tract.

4 hours after copulation

A small quantity of sperm now lies in the oviducal side of the common duct, near the junction with the carrefour (Fig. 399). The carrefour remains empty.

6 and 8 hours after copulation

The reproductive tract appears normal. A large volume of sperm is stored within the hermaphrodite duct. The remains of the spermatophore fill the bursa copulatrix. No sperm is seen in the carrefour.

EGG-LAYING

Since it has proved impossible to predict the timing of oviposition, observations on egg-laying animals are restricted to the few individuals who began producing eggs while being cleaned and fed. This occurred very infrequently and the following descriptions are based on information from only three D.reticulatum and seven A.hortensis.

Egg-laying in D.reticulatum

No spindles were seen in the gonadal oocytes and maturation divisions were first observed in oocytes passing through the seminal vesicle (Fig. 400).

The oocytes become distorted as they enter the slender coiled portion of the hermaphrodite duct but resume their spherical shape upon entering the pouched diverticulum of the carrefour (Fig. 401). The flattened pouch swells as the number of oocytes increases until it is completely distended and bursts easily on handling. The finger-like diverticulum remains empty, however, and maintains its normal configuration (Fig. 401). Consequently, the inner epithelium of the pouch remains columnar, while the rest becomes squamous, with only a very thin layer of connective tissue (Fig. 402). Although spindles were present in many of the oocytes

(Fig. 403), no polar bodies were seen. A small quantity of sperm also enters the carrefour, but remains near the entrance of the hermaphrodite duct (Fig. 404).

Throughout egg-laying the albumen gland is very active and the collecting ducts are swollen with secretion (Fig. 405). The eggs become coated with albumen as they pass through into the common duct. They occupy the oviducal lumen, and as they continue anteriorly towards the genital opening they receive secretions from the oviducal secretory cells (Figs. 406 & 407). Spindles are occasionally seen in oocytes which have been coated with albumen and which are receiving the jelly and outer shell layers (Fig. 408).

Egg-laying in A.hortensis

At the onset of egg-laying spindle bodies develop in many of the vitellogenic oocytes (Figs. 409 & 410). The follicle cells then rupture releasing these oocytes into the lumina of the gonadal acini (Fig. 411). The efferent ductules are very slender, only 10 to 15μm in diameter. Consequently, the large oocytes (diam. 20-30 μm) elongate as they pass through the ductules (Fig. 412) and only resume their normal spherical appearance when they enter the hermaphrodite duct (Fig. 413). Small, pre-vitellogenic oocytes (diam. <5μm) may be released at the same time. They either form aggregates (Fig.

414) or continue along the reproductive tract as isolated cells.

The eggs pass through the mass of stored sperm in the hermaphrodite duct, and accumulate at the base and in the ascending limb of the carrefour loop (Figs. 415-418). As the number of oocytes increases the duct expands. The epithelial cells become very stretched and eventually they appear squamous rather than columnar. In animals just starting to lay, up to fifty oocytes may be stored in the carrefour. Some sperm may also pass into the carrefour loop along with the oocytes (Fig. 419).

Here, most of the oocytes complete their maturation divisions and spindles are commonly seen associated with one, or occasionally two, polar bodies (Figs. 420 & 421). In some animals maturation is further advanced, the spindles are absent and two clear patches have developed within the mature ovum. These correspond to the sperm and egg nuclei (Figs. 422 & 423).

The albumen gland is actively secreting throughout egg-laying and as in D.reticulatum the collecting ducts become distended with secretion. As the fertilized ova pass out of the carrefour they become coated with albumen resulting in a tenfold increase in diameter. Excess albumen fills the proximal part of the male

groove. The eggs continue through into the common duct and pass along the oviducal side of the duct. The oviducal gland is swollen with secretion and this is gradually added to the eggs forming the jelly and then the outer shell layers (Fig. 424). In some animals the polar bodies are emitted after the addition of the albumen (Fig. 425).

The eggs pass directly into the free oviduct. They are no longer easily distorted and the duct itself expands to accomodate them. The slender region of the oviduct stretches considerably, so that the epithelium becomes squamous and the whole duct appears as a fine line surrounding the egg (Fig. 426). The thicker, anterior half of the oviduct has a greater capacity for expansion and although the epithelium becomes squamous the muscle layers are still discernable (Fig. 427).

The eggs eventually pass into the atria (Figs. 424l & 428), which also expand, before being extruded through the genital opening.

The results of histochemical tests on the layers investing the fully-formed eggs can be seen in Table 16.

Table 16 Results of the histochemical tests on the egg layers

(i) D. reticulatum

Egg layers	P.A.S. (carbo-hydrates)	Alcian Blue (acid muco-polysaccharides)	Bromophenol blue (proteins)
Perivitelline fluid	+++	-	++
Inner jelly layer	++	++	-
Outer jelly layer	+	+	-
Shell layer	+	+	-

(ii) A. hortensis

Egg layers	P.A.S. (carbo-hydrates)	Alcian Blue (acid muco-polysaccharides)	Bromophenol blue (proteins)
Perivitelline fluid	+++	-	++
Inner membrane	+++	++	-
Inner shell layer	++	++	-
Outer shell layer	-	+	-

+++ Strongly positive

++ Positive

+ Weakly positive

- Negative

DISCUSSION

Courtship

Prolonged courtship procedures have been described for many slugs, including D. reticulatum (Gerhardt, 1934, 1935; Carrick, 1938; Quick, 1960; Karlin and Bacon, 1961; Webb, 1961; Newell, 1966; Runham and Hunter, 1970) and A. hortensis (Gerhardt, 1935; Quick, 1946, 1960; Davies, 1977). Possibly the most elaborate is the aerial courtship of Limax maximus (Gerhardt, 1934; Chase, 1953), but however spectacular, the same basic elements are featured repeatedly in the slugs' courtship behaviour.

(1) "Following" - When a slug is ready to mate its locomotory mucus becomes interesting to other receptive individuals. Those crossing such a trail will immediately change course and start to pursue the leading animal. (Gerhardt, 1934, 1940; Quick, 1947, 1960; Newell, 1966; Runham and Hunter, 1970).

Since copulation normally occurs at night location of a partner cannot rely on any optical information. The mucus, presumably, contains some factor which indicates an individual's readiness to mate. The "following" slug frequently eats the mucus trail (Barr, 1928; Davies, 1977) which suggests that

these animals use a combination of olfactory and gustatory cues. In this way two receptive individuals can come together and do not have to depend upon chance encounters.

(2) "Pairing" - The two animals take up their copulatory positions, the head of one facing the tail of the other. Gerhardt (1936) suggests that this position arose in the more primitive pulmonates where a shell limits access to the genital opening.

(3) "Circling" - Throughout courtship the pair circle slowly in a clockwise direction. At first the animals may remain a little apart, but gradually they come closer together until the genital openings are apposed (Taylor, 1907; Gerhardt, 1934, 1935; Mead, 1942; Quick, 1960; Karlin and Bacon, 1961; Duncan, 1975). The Arionidae tend to circle very slowly and some pairs become stationary after the initial contact (Gerhardt, 1940; Quick, 1947).

"Circling" is accompanied by "caressing," "biting" and the production of copious mucus.

(4) "Caressing" - Many slugs caress one another during courtship. Some species, including Limax maximus simply use their tentacles (Chase, 1953), while Milax and Deroceras have specially adapted stimulators which can be protruded through the genital opening (Quick, 1960). The Arionidae appear to require no external stimulation and only caress one another during the final stages of copulation (Quick, 1946, 1960).

(5) "Biting" - Members of the Limacidae appear to bite one another during courtship. (Mead, 1942; Karlin and Bacon, 1961; Runham and Hunter, 1970; Duncan, 1975). The head is thrust forward with the radula exposed, and is usually directed towards the protruded stimulatory organs. The animals may be eating the mucus which is produced at this time. Alternatively, rasping the radula over sensitive areas of the body may have a stimulatory effect.

Although there are no published reports of "biting" amongst the Arionidae, adult A.ater are commonly seen with white, circular lesions on their bodies. It is generally assumed that these lesions are the result of aggressive courtship behaviour (Runham,pers.comm.).

(6) "Tentacle withdrawal" - Occasionally, the tentacles are withdrawn immediately prior to copulation (Adams, 1898; Mead, 1942; Quick, 1946; Karlin and Bacon, 1961). This may increase the internal pressure and thus aid eversion of the anterior reproductive tract or ejection of the sperm mass. It does not occur at every mating but the position of the head often hides the tentacles from view, which could account for the limited number of observations.

Detailed experiments on the courtship and copulatory behaviour of H. pomatia (Jepperson, 1976; Lind, 1976) and several species of Octopus (Wells and Wells, 1972) have shown that there is no simple chain of events where one sequence of behaviour initiates the next. These authors suggest that individual behavioural elements are integrated and controlled centrally, by the nervous and/or endocrine systems, and it seems likely that this is true for other molluscs, including the slugs.

Copulation in D.reticulatum

The sequence of events observed in fixed specimens is reproducible and follows a strict chronological pattern. This suggests that the timing of the sperm's release from the hermaphrodite duct and its subsequent progress along the reproductive tract is fairly constant.

Preparation of the sperm package takes 30 to 40 minutes. This agrees with the above descriptions of mating behaviour which recorded courtships lasting between 0.5 to 1.5 hours. Pairs seen circling for shorter periods never exchanged their sperm. It is rare, however, for slugs to copulate immediately and the completed sperm package may be retained within the penial sac for a further one hour. The reason for the prolongation of the later stages of courtship is not known, but full eversion of the penial sac may require additional preparations which cannot be detected histologically.

Several questions concerning the functioning of the reproductive tract, during and immediately after courtship and copulation, have arisen from these observations.

Spermatozoa only pass into the slender portion of the hermaphrodite duct during early courtship. At all other times the large volume of stored sperm remains within the seminal vesicle and some chemical or physical barrier prevents it flowing into the rest of the reproductive tract.

The sperm are densely packed and appear to be incapable of autonomous movement. Some authors suggest that passage through the reproductive tract is essential for sperm capacitation (Thompson and Bebbington, 1969; Bayne, 1973) and only when it has been transferred at copulation is it able to rely on its own propulsion. Inactivation of the sperm, however, may simply be due to the viscosity of the surrounding fluid. This has been observed in mammals, where the presence of a large glycoprotein increases the fluid's viscosity and mechanically inhibits sperm movement (Usselman and Cone, 1983).

One possible physical restriction on the free movement of sperm is the sudden narrowing of the duct as it nears the carrefour. At the same time the epithelium becomes completely ciliated and this may effectively seal the proximal end of the seminal vesicle, and so prevent the passive outflow of sperm.

Upon dissection of the hermaphrodite duct the sperm flows freely from both the cut ends. Addition of adrenaline (adrenaline acid tartrate, 1.8 mg/ml) will coagulate the fluid sperm and stimulate the duct to contract rhythmically along its length, expelling gelled sperm anteriorly. This suggests a possible mechanism for controlling sperm release:

The sperm may be stored in a fluid state, but remains incapable of autonomous movement. If adrenaline or an adrenaline-like substance is released at the onset of courtship this may stimulate peristalsis in the hermaphrodite duct, and at the same time affect the fluidity of the sperm. A more solid mass is possibly moved along more easily. Positive pressure from the sperm mass would then be sufficient to overcome the ciliary check on passive movement, and sperm would flow from the seminal vesicle towards the carrefour. Regular beating of the cilia may further assist this forward movement.

It has been suggested that a massive discharge from the secretory epithelial cells, combined with ciliary agitation, flushes the sperm mass through the hermaphrodite duct (Runham, 1984), but there is no evidence to support a sudden and copious release of secretion (p. 235).

The flow of sperm is checked as soon as it reaches the carrefour and, again, some physical or chemical barrier must prevent it flowing straight through into the common duct. There are several possible explanations for this. Contraction of muscles at the base of the carrefour could close off the anterior portion of the reproductive tract; the ciliary beat may be reversed at this level; the initial stream of sperm may react with secretions from the albumen gland, carrefour gland or both, to form a plug which temporarily impedes the sperm's progress; or it may be a combination of these and other factors. There is no evidence that the duct becomes constricted when the pouched diverticulum fills with sperm, which tends to exclude the theory of muscular contraction. Since ciliary beat and the formation of a plug cannot be demonstrated histologically, the temporary halt in sperm movement remains a problem.

While the sperm is retained within the carrefour's pouched diverticulum the prostate gland starts to release its secretion. The stimulus to release may be coordinated by stretch receptors in the extensive nerve net which surrounds the carrefour.

Experiments on vivisected slugs demonstrated that ciliary currents in the male groove transported particles of Indian ink rapidly towards the vas deferens

(Bayne, 1967). It seems likely, therefore, that prostate secretion and, probably, sperm are moved along in this way.

As the sperm enters the vas deferens it appears to be packaged into discrete bundles, surrounded by secretion from the prostate gland. Such packaging would be achieved by peristaltic contraction of the duct, ejecting sperm, spasmodically, into the vas deferens. Minker and Koltai (1961) report peristalsis in the freshly dissected vas deferens of Helix pomatia and Limax maximus, and the thick muscle sheath present in D.reticulatum suggests that regular contraction of this duct conveys the sperm into the penial sac.

The function of the trifid appendage is obscure. Its position is comparable to the flagella of Testacella haliotidea (Quick, 1960), the Helicidae (Kilias, 1960) and the Bulimulidae (Mol, 1971). Unfortunately, there are no reports of copulation in the slug Testacella and the mechanism of sperm transfer is unknown. In H.pomatia however, the tail of the spermatophore develops within this flagellum and its structure plays an important role in the sperm's release (Lind, 1973).

Comparative studies of closely related pulmonates may reveal an evolutionary trend in the contribution played by the flagellum, trifid appendage or other

analagous diverticula. In D.reticulatum the sperm package is seen in its simplest form, the sperm surrounded by the gelatinous secretions of the prostate gland, and for this reason it is frequently known as the jelly mass (Runham and Hunter, 1970). Taylor (1907) postulated that this is, in fact the precursor of the spermatophore. If this is so, the prostate secretion in the trifid appendage may represent the rudimentary tail of the spermatophore. If, on the other hand, the jelly mass is a highly specialized, rather than primitive, form of the sperm package, the involvement of the trifid appendage may echo an earlier more important contribution. It is significant to note that type C prostate secretion, abundant in the trifid appendage, appears to be absent from the sperm package.

The jelly mass assumes the basic shape of the penial sac but does not appear to take on the fine contours of the ridged epithelium. Its soft nature and the presence of cilia on the epithelial cells probably prevent such superficial detail persisting. It has been suggested that, in addition to packaging, the prostate secretion provides nutrition, and may play a role in activating the sperm, thus assisting its passage towards the carrefour after copulation (Bayne, 1967; Els, 1978).

Half the supply of stored sperm is mobilized during courtship and fresh sperm from the gonad replenishes stocks in the seminal vesicle within forty minutes of protruding the sarcobellum. This is an interesting feature, demanding controlled shedding of mature spermatozoa from the Sertoli cells, which implies that some hormonal factor is involved.

Transference of the sperm package at copulation is now clearly understood and its subsequent transport to the site of fertilization can be postulated.

When both slugs are ready to copulate muscular contractions in the body wall pump blood anteriorly and the rising pressure forces the penial sac out of the genital opening (Figs. 429b & c). As the pressure increases the penial sac dilates and the blood sinus in the core of the sarcobellum becomes suffused with blood. Eversion of the penial sac instantly transports the sperm package to the exterior, and the oviduct and bursa copulatrix now open directly into a slit-like groove which forms between the penial mass and the body wall (Fig. 429c). The penial sacs interlock, and rapid, clockwise circling of the animals around their penial masses conveys the sperm along the groove towards the bursa copulatrix (Fig. 429d). The fusiform shape and flexible nature of the jelly mass must optimize this

type of transfer, since a longer, more rigid spermatophore would be unmanageable.

The tip of the sperm package lodges in the entrance to the bursa and remains in this position when the penial mass is withdrawn. Gradually the sperm package is drawn into the bursa sac (Figs. 429e - g) by regular peristaltic contractions of the bursa duct (Lind, 1973), and within 24 hours it has started to break down. Excess sperm is eventually digested and resorbed through the epithelium.

A small amount of sperm remains near the base of the trifid appendage and appears to enter the vas deferens (Fig. 429g). Although there is no direct evidence to confirm the sperm's journey along the common duct, it is reasonable to assume that this very small quantity of foreign sperm travels along the vas deferens and male groove towards the carrefour, where it is temporarily stored in the smaller of the two diverticula. During the next few hours it passes into the slender portion of the hermaphrodite duct and then into the seminal vesicle, where it is stored together with the animal's own sperm. Possibly, the sperm at the anterior end of the hermaphrodite duct preferentially fertilizes the oocytes as they pass into the carrefour.

It is very likely that some of this newly received sperm is transferred to a third animal at a subsequent mating. Such "sperm-sharing" has been demonstrated recently in Biomphalaria glabrata (Monteiro et al., 1984).

Copulation in A.hortensis

Many of the points discussed for D.reticulatum also apply for A.hortensis, e.g. release of sperm from the hermaphrodite duct, temporary storage of sperm in the carrefour and movement of sperm along the reproductive tract. However, in A.hortensis comparatively little sperm is released at each mating and the hermaphrodite duct is not obviously emptied. Consequently, the highly organized shedding of sperm from the Sertoli cells is not observed. In addition, the descending limb of the carrefour can easily accomodate the whole volume of sperm without having to expand, so the proposed involvement of stretch receptors coordinating activity cannot now apply. There is no discrete packaging of the sperm as it enters the vas deferens, and a steady stream of the sperm/prostate mixture appears to flow directly into the epiphallus. Here the spermatophore is finally formed.

As in D.reticulatum, the timing of the sperm's movement along the reproductive tract follows a consistent chronological pattern. The behavioural observations, however, have recorded courtships lasting only two hours and some animals must copulate as soon as the sperm reaches the epiphallus. At this stage the spermatophore is still incomplete and will disintegrate upon dissection. These premature matings are probably unsuccessful. More usually, the sperm is retained in the epiphallus for up to two hours. During this time the secretions harden (Smith, 1966) and may assume the shape of the papillated epithelium.

Spermatophores removed at copulation vary in their complexity. Some resemble cloves (Fig. 430a), their anterior end forming a flattened disc, which is crenellated around the edge, while a ridge of approximately 56 forward pointing serrations runs the whole length of the convex side (Quick, 1946, 1960). Others have a much simplified structure (Fig. 430b), lacking both disc and serrations (Gerhardt, 1935; Quick, 1960). These differences may be regional, with whole populations favouring one form or the other (Quick, 1960). Alternatively, the degree of complexity may be related directly to the length of time the sperm remains within the epiphallus. Longer courtships would be advantageous, therefore, since the surface architecture

appears to assist in attaching the spermatophore at copulation.

Exchange of spermatophores is completely unlike the transference of jelly masses seen in D.reticulatum. When both slugs are ready to exchange their sperm muscular contractions in the body wall pump blood anteriorly, and the rising pressure forces the atria out through the genital openings (Fig. 431b). At first the upper atrium is only partially everted but this is sufficient to expose the glandular cone of the epiphallus. The pair are positioned so that both cones can be inserted simultaneously into the channels which lead towards the bursae. The entrances to these channels take on the shape of the epiphallic cones and the two animals become temporarily united (Fig. 431d). A final convulsive spasm completes the inversion of the upper atrium and as it expands, it elongates, forming a finger-like structure which extends over the back of the partner (Fig. 431c).

Previous workers (Quick, 1960) had assumed that the oviduct itself was everted to form this elongated structure, but close histological examination reveals no evidence to support this. In the first place, the sub-epithelial muscle is obliquely orientated. This arrangement is characteristic of the upper atrium rather than the oviduct, which is surrounded by concentric

layers of circular muscle. Secondly, eversion of the oviduct would effectively reduce the length of the normally orientated duct. Comparison of the oviduct at rest and during copulation revealed no such changes in length.

The thick, muscular oviduct runs through the centre of the everted atrium, opening near the apex. Its solid structure may now support the soft-walled atrium and therefore dictate its final shape.

The spermatophore is now a hardened structure with a ridge of surface spines and needs to pass out of the epiphallus, through the centre of the epiphallic cone. This can only be achieved by the duct peeling away from the solid spermatophore, a process comparable to the removal of a rubber mould from a plaster cast. As the epiphallus steadily turns inside out it pushes the spermatophore towards the closely apposed entrance of the partner's bursa copulatrix.

As in D.reticulatum the anterior tip of the sperm package lodges in the entrance of the partner's bursa and remains in this position when the atria are withdrawn. Most of the spermatophore passes directly into the bursa sac, but this sperm cannot survive and only that which completely avoids the bursa contents is capable of fertilizing the ova. The mechanism of sperm

release may resemble that of Helix pomatia . In this snail the outer shell of the spermatophore is prepared during courtship but the sperm is only added at the start of copulation. Consequently, while the anterior portion is completely sealed, the tail remains open, and as the spermatophore is drawn into the bursa, a large volume of sperm is expressed from the posterior opening and into the oviduct (Lind, 1973). Although in A.hortensis the formation of the spermatophore and its final structure are very different to that of H.pomatia, the basic principle of sperm release may be similar. Alternatively, Quick (1946) suggests that the ridge of serrations facilitates longitudinal rupture of the spermatophore, releasing sperm directly into the upper atrium.

Throughout copulation the gland cells of the lower atrium secrete continuously. This secretion bathes the everted organs and on withdrawal it fills both the atria and oviduct. Since the oviduct itself does not evert, the presence of secretion there may result from the changing internal pressures. This lipid secretion may function as a lubricant.

Only a small amount of sperm passes up the reproductive tract, travelling along the oviducal side of the common duct towards the carrefour. Since no free sperm was observed four hours post-copulation, it

probably passes directly into the seminal vesicle where it is stored until required for fertilization or the formation of another spermatophore.

The function of the bursa copulatrix

Research into the fate of the sperm immediately after copulation has made it necessary to continually revise the role of the bursa copulatrix in pulmonate reproduction.

In many species the sperm package is inserted directly into the entrance of the bursa at copulation. Regular peristaltic contractions of the bursa stalk draw this sperm into the saccule, which eventually becomes filled with the sperm and packaging material.

Early workers assumed that sperm was only released from the spermatophore when the hardened, outer layers had been dissolved by the bursal secretions (Meisenheimer, 1907; Bretschneider, 1948). Repeated observations of sperm in the bursal sac led many to believe that this was the storage site for foreign sperm (Baudelot, 1863; Hoffmann, 1923; Ikeda, 1937; Holm, 1946; Cain, 1955; Duncan, 1958, 1960), and it was suggested that the sperm underwent various modifications before it was capable of further migration and fertilization of the ova (Baudelot, 1863; Ikeda, 1937;

Bretschneider, 1948; Duncan, 1958; Els, 1973; Sirgel, 1973; Stears, 1974). Gradually, however, it was realized that the sperm showed signs of degeneration within just a few days of receipt (Boettger, 1944; Alaphilippe, 1955; Cain, 1955). Prolonged storage, therefore, was impossible and it became generally accepted that the bursa was only a temporary store, with the foreign sperm accumulating elsewhere during the period between copulation and fertilization.

Subsequent work has now revealed that although a large proportion of the sperm enters the bursa, only that which bypasses the bursa sac has any chance of further migration along the reproductive tract (Lind, 1973). In H.pomatia only about 0.1% of the sperm received at copulation actually arrives in the spermatheca (= carrefour diverticulum) (Lind, 1973).

The bursa copulatrix is now considered to be a digestive organ, responsible for the removal of superfluous foreign sperm, the animal's own excess sperm and other waste products of reproduction (Alaphilippe, 1955, Rigby, 1963; Fretter and Graham, 1964; Lind, 1973), and histochemical investigations have demonstrated the presence of enzymes such as ribonuclease, deoxyribonuclease and hydrolases (Németh and Kovács, 1972; Reeder and Rogers, 1979).

Strong, peristaltic contractions of the bursa stalk ensure that everything leaving the reproductive tract, apart from the large, fertilized eggs and fully formed sperm package, is transported into the bursa, and to date there is no evidence that any material leaves again through the stalk (Lind, 1973). Rigby (1963) suggests that the breakdown products are taken up by the bursal epithelial cells and possibly re-utilized. In many species the bursa darkens with age, probably due to the accumulation of undigested residue (Cain, 1955; Lind, 1973).

Continual revision of the role played by the bursa copulatrix in pulmonate reproduction has led, inevitably, to a variety of names all referring to the same homologous organ (Table 17). The "spermatheca" and "seminal receptable" (= "receptaculum seminis") both imply sperm storage. This is now a misnomer and its usage is gradually decreasing, becoming replaced by the term "bursa copulatrix" or "copulatory pouch". Thompson and Bebbington (1969) have argued that since this bursa does not receive the penis at copulation, but mainly digests and destroys the stray gametes, it should be renamed the "gametolytic gland". Common usage has not followed their suggestion, however, and the majority of workers still use "bursa copulatrix".

Table 17

Synonyms for the bursa copulatrix and examples of usage

Bursa copulatrix (= copulatory pouch)

Creek	1953	Pomatia elegans
Fretter	1953	Prosobranchs in general
Alaphilippe	1955	Planorbarius corneus
Duncan	1958	Physa fontinalis
Lind	1973	Helix pomatia
Visser	1973	Gonaxis gwandaensis
Bishop	1978	Sphaerospira fraseri
Runham	1978	Deroceras reticulatum

Spermatheca

Dasen	1933	Ariophanta ligulata
Horstmann	1955	Lymnaea stagnalis
Quick	1960	Pulmonate slugs in general
Bayne	1973	Succinea grosvenori, S.unicolor
Noyce	1973	Theba pisana
Sirgel	1973	Agriolimax caruanae
Els	1974	Milax gagates
Stears	1974	Limax valentianus
Evans	1978	Limax pseudoflavus

Seminal receptacle (= receptaculum seminis)

Ikeda	1937	Philomycus bilineatus
Holm	1946	Lymnaea stagnalis
Forcart	1948	Phenacolimax major
Cain	1955	Lymnaea stagnalis
Kilias	1960	Helix pomatia
Németh and Kovács	1972	Helix pomatia

Gametolytic gland

Runham	1984	Molluscs in general
Thompson and Bebbington	1969	Aplysia

The passage of eggs through the posterior region of the reproductive tract

In the gonad the oocytes develop in the walls of the acini. They are isolated from the male gametes in the acinar lumen by a closely applied layer of follicle cells, which are, in turn, overlaid by Sertoli cells (Hogg and Wijdenes, 1979). As vitellogenesis proceeds a follicular cavity develops between the follicle cells and the oocytes, while microvilli appear on the oocyte membrane.

It is generally believed that muscular contraction of the acinar walls ruptures both the follicle and overlying Sertoli cells, and releases the oocytes into the lumen of the gonad (Coggeshall, 1972). Saleuddin and Khan (1981), however, now propose that the oocytes leave the follicular sheath by independant amoeboid movement. They argue that generalized muscular contraction of the acinus would have to rupture the junctional complexes present between the oocyte and follicle cells, and would not distinguish between young and ripe oocytes. The most convincing evidence, however, comes from their *in-vitro* experiments on *Helisoma* oocytes, which change shape in response to cerebral ganglia homogenates, probably containing an ovulation hormone. Early reports of amoeboid movement

by planorbid ova (Merton, 1924, 1926, 1927) add further evidence to this theory.

Once in the ciliated duct of the acinus, muscular contractions and cilary movement probably propel the oocyte into the hermaphrodite duct. Here, the oocytes must pass through the mass of densely packed sperm, stored in the seminal vesicle. Peristalsis would inevitably lead to large numbers of sperm being expelled from the hermaphrodite duct, together with the oocytes. This was not observed, and there must be some other means of propulsion. Ciliary movement cannot be effective since the oocytes pass through the centre of the sperm mass. The possibility that oocytes rely on independent amoeboid movement cannot be ignored, although the in vitro experiments performed by Saleuddin and Khan (1981) suggest that this is a slow process, and only suitable for short distances. Since it proved impossible to stimulate egg-laying during this investigation, the time between ovulation and the oocytes arriving at the carrefour, is still unknown, and therefore cannot give any indication of the mechanism involved.

As soon as the oocytes leave their follicular sheaths they are exposed to the male gametes. Those in the gonad are mostly immature, since the spermatozoa pass into the hermaphrodite duct immediately after

maturation (Aubry, 1954). Those in the seminal vesicle, however, may be capable of fertilizing the oocytes. The incidence of self-fertilization will be discussed, in full, later.

The function of the carrefour

Although there are numerous reports concerning the morphology of the carrefour its function has been less thoroughly investigated. Many authors have postulated roles for the various diverticula and this has led to considerable confusion in carrefour terminology (Table 18).

As early as 1907, Meisenheimer observed fertilized oocytes within the large flattened saccule of Helix pomatia . He concluded that this was the site of fertilization and named it the "Befruchtungstasche" (= fertilization chamber or pouch). Since then a number of authors have reported oocytes undergoing maturation and fertilization within this chamber (Perrot, 1937; Gugler, 1964). Usually all the ova are at the same stage of development and it is rare to see a sequence of events in a single, fixed animal.

It was generally assumed that the central diverticulum functioned as a spermatheca (= seminal receptacle), storing the foreign sperm until required

for fertilization (Perez, 1868; Meisenheimer, 1907; Dasen, 1933). There have been many reports of sperm being present in this region but difficulties arise when it comes to distinguishing foreign sperm, received at copulation, from the animal's own stocks. Several workers observed that the spermatozoa stored in these sacs were orientated, their heads directed towards the epithelium (Gugler, 1964; Rigby, 1963, 1965; Mol, 1971; Lind, 1973), and Lind (1973) and Bayne (1973) further demonstrated that orientated sperm was only present after copulation. This sperm remained viable for over one year. Thus, the terminology of fertilization pouch and spermatheca appears to be well founded.

In those slugs where separate carrefour diverticula are absent the ascending limb of the carrefour loop functions as a fertilization chamber (Ikeda, 1973). Ikeda observed that in Phylomycus bilineatus the oocytes enter this chamber before rupture of the germinal vesicle. Here they undergo maturation, fertilization and, occasionally, their first cleavage, but although the oocytes fill the duct in an orderly sequence, the oldest lying anteriorly near the junction with the albumen gland, there is again a high level of synchrony in their development.

Ikeda believed that foreign sperm was stored in the bursa copulatrix, only migrating up towards the carrefour when newly ovulated eggs required fertilization. It has since been shown that the bursa is a digestive organ, concerned with the removal of reproductive waste (Lind, 1973) and it is likely that a small volume of sperm (0.1% of the total sperm mass in H.pomatia, Lind, 1973) passed up the reproductive tract unnoticed. The storage site of foreign sperm in this type of carrefour has, therefore, not previously been discussed.

In the present study oocytes have been observed in the "fertilization chambers" of both D.reticulatum and A.hortensis and here they undergo maturation and possibly fertilization. Although there is synchronous development of the ova, the timing of maturation is very variable. In the few animals examined it appeared to proceed more rapidly in A.hortensis than D. reticulatum, but this variation may not persist when larger samples are studied.

In Arion maturation divisions were frequently seen in gonadal oocytes, and fusion of the sperm and egg nuclei usually occurred before the oocytes left the carrefour. In contrast, meiotic spindles were not observed in D.reticulatum until the oocytes were passing through the seminal vesicle, and the absence of

polar bodies suggests that the first maturation divisions had not beem completed by the time the oocytes were coated with albumen. Despite the immaturity of the oocytes, sperm must penetrate before the addition of vitellogenic fluid. Possibly, the sperm head remains near the periphery of the oocyte cytoplasm until both oocyte maturation divisions are completed and only then will the sperm head swell, shorten and vacuolate. This sperm nucleus now fuses with the egg nucleus to form the zygote. If the maturation divisions occur after the albumen has been added, one or two polar bodies will remain fixed to the surface of the zygote and this was seen in an individual A.hortensis.

By delaying fusion of the gamete nuclei the timing of oocyte maturation is not critical and successful fertilization does not depend upon mature ova being in the right place at the right time. As mentioned before, however, the fertilizing sperm must penetrate the oocyte before the albumen has been added. Following copulation a very small quantity of foreign sperm travels up the reproductive tract towards the carrefour.
In D.reticulatum this sperm only pauses briefly in the spermatheca before continuing along the narrow hermaphrodite duct to the seminal vesicle. Similarly, in A. hortensis foreign sperm appears to be stored in the hermaphrodite duct.

In both species very little sperm is seen in the fertilization chamber. This may be the small quantity of stored, foreign sperm which has now travelled back into the carrefour, but it seems unlikely that this is sufficient to fertilize every oocyte. Perhaps fertilization occurs in the anterior region of the seminal vesicle. The sperm here may not experience the same inhibition of movement as sperm further in the hermaphrodite duct, and since this appears to be the storage site for foreign sperm there would be preferential cross-fertilization. But, if fertilization has already taken place why are the oocytes retained in the so-called fertilization chamber? It may act as a reservoir, controlling the flow of oocytes and ensuring that they receive the correct amount of albumen before continuing on into the common duct. This area is certainly highly innervated and appears to be a co-ordinating centre for gamete movement.

The number of oocytes present in the fertilization chamber (40 - 50) greatly exceeds the number of eggs actually deposited (av. clutch size: D. reticulatum, 23; A.hortensis, 28). This has been observed in other stylommatophoran snails, an extreme example being Strophocheilus oblongus where around 200 ova are fertilized but an average of only 4 eggs are laid (Tompa, 1984). The excess oocytes presumably continue through the reproductive tract, without

receiving the perivitelline and egg shell layers, and upon reaching the genital atrium peristalsis of the bursa duct conveys these oocytes into the digestive saccule (Lind, 1973; Tompa, 1984). If the capacity of the albumen gland dictates the number of eggs deposited, the uncoated oocytes are those left when the supply of perivitelline fluid is exhausted. Alternatively, the epithelial cells lining the fertilization chamber may phagocytose the redundant oocytes. This would parallel the disposal of sperm in the hermaphrodite duct and could account for the age related accumulation of lysosomes.

Table 18

Synonyms for the carrefour diverticula in the Stylommatophora and examples of usage

Fertilization chamber/pocket/pouch/sac

Meisenheimer, 1907	Helix pomatia
Hoffmann, 1920	Limax maximus
Baecker, 1932	Limax cinereus, Arion empiricorum
Ikeda, 1937	Philomycus bilineatus
Kilias, 1960	Helix pomatia
Duncan, 1960a	Pulmonates in general
Kugler, 1965	Philomycus carolinianus
Flasar, 1967	Oxychilus draparnaudi
Els, 1974	Milax gagates

Vesicula seminalis/seminal vesicle

Baudelot, 1863	Limax cinereus
Brock, 1884	Agriolimax agrestis
Simroth, 1887	Agriolimax laevis
Taylor, 1907	British land and freshwater molluscs
Pabst, 1914	Arion empiricorum
Quick, 1960	Pulmonate slugs in general
Duncan, 1961	Succinea pfeifferi
Lüsis, 1961	Arion ater rufus

Receptaculum seminis/seminal receptacle/spermatheca

Dasen, 1933	Ariophanta ligulata

Fertilization chamber-receptaculum seminis/fertilization chamber spermatheca complex

Rigby, 1963	Oxychilus cellarius
Rigby, 1965	Succinea putris
Lind, 1973	Helix pomatia
Noyce, 1973	Theba pisana
Sirgel, 1973	Agriolimax caruanae
Stears, 1974	Limax valentianus

Caecum

Reynell, 1906	Burtoa nilotica

Talon

Mead, 1942	Ariolimax spp
Mead, 1950	Achantinidae
Berry, 1963b	Gyliotrachella depressispira
Ghose, 1963	Achatina fulica
Gugler, 1964	Anguispira alternata
Mol, 1971	Bulimulidae
Owiny, 1972	Achatinaceae
Bishop, 1978	Sphirospira fraseri

The passage of eggs through the anterior region of the reproductive tract

The common duct has been described by numerous authors and the histochemistry of its various secretions has been thoroughly investigated, particularly in relationship to the formation of eggs (e.g. Smith, 1965 - Arion ater; Bayne, 1967 - D.reticulatum).

The nutritive layer of the egg is derived solely from the albumen gland. It is surrounded by the perivitelline membrane which forms a sac, strong enough to maintain the ovoid shape after the egg shell layers have been removed (Bayne, 1966). Following analysis by thin layer chromatography, Bayne (1966) concluded that this perivitelline membrane is formed by coagulation of the surface albumen rather than addition of a separate secretion. Subsequent studies have cast doubt on this theory. Amino acid analyses show raised levels of arginine and cysteine in the perivitelline membrane of Strophocheilus oblongus (Tompa et al., 1977) while a recent histochemical investigation on the eggs of Achatina fulica clearly demonstrates the different nature of perivitelline membrane and fluid (Ramasubramaniam, 1979).

Solem (1976) proposed that in Anguispira alternata the perivitelline membrane is secreted by a special gland (= perivitelline membrane gland) which opens into the uterus near the junction with the carrefour. In many Stylommatophora the secretory cells lining this region of the oviducal groove are morphologically different to those lining the rest of the duct, and have been variously named the posterior nidamental gland (Kugler, 1965; Sirgel, 1973; Stears, 1974) the oviducal gland (Els, 1974), and the mucous/ muciparous gland (Rigby, 1963; Noyce, 1973). All these may be functionally comparable to the perivitelline membrane gland described by Solem.

The supporting layers are added as the egg passes along the ccommon duct. In D.reticulatum a jelly-layer is surrounded by the egg-shell membranes, the jelly nearest the perivitelline membrane appearing denser, but histochemically identical to the rest of the gel layer. Both layers contain calcium in a carbohydrate matrix, and the level of calcium increases towards the periphery (Bayne, 1968b). Since the embryo obtains most of its calcium from the perivitelline fluid, high levels remain in the jelly and shell layers after hatching. This calcium may contribute to the physiological buffer necessary to protect the embryo from the external environment (Bayne, 1966), and Bayne further suggests that while the jelly provides a short-term reservoir of

water for the egg, the shell provides mechanical support.

The jelly layer is secreted by the posterior portion of the oviducal gland while the shell layers are added anteriorly, and Bayne (1967) demonstrated histochemically that the secretory nature of this gland changes accordingly.

The eggs of A.hortensis are similar to those described for Arion ater (Bayne, 1968b). A thin layer of P.A.S./alcian blue-positive material is closely applied to the perivitelline membrane. This is surrounded by two thicker layers. The inner layer stains strongly with P.A.S. while the outer layer remains clear, which agrees with the staining characteristics of the two shell layers of A.ater, rather than the jelly and shell layers of D.reticulatum (c.f. Bayne, 1968b). In A.ater the outermost shell layer is heavily calcified and coated in lipid (Bayne, 1966). The shell layers are secreted by oviducal gland cells but the lipid coat appears to be added by the "spongy gland", as the egg passes out through the lower atrium. Since the reproductive tract of A.hortensis closely resembles that of A.ater, with the same lipid-rich gland associated with the lower atrium, the eggs of A.hortensis may be similarly coated. The function of this lipid layer is not clear since it

does not improve the eggs' resistance to desiccation (Bayne, 1966).

Tompa and Wilbur (1977) demonstrated that in H.aspersa the level of calcium in the haemolymph rises during egg-formation. Since there is no calcium stored in the reproductive tract of these snails they concluded that calcium is mobilized from the shell and transported via the blood to the common duct. The Arionidae have no discrete shell but the blood vessels of A.ater are heavily calcified (Duval, 1981), and in A.hortensis the papillae of the epiphallus are packed with calcium cells. In D.reticulatum , where a small shell is enclosed within the mantle, there appear to be limited stores of calcium. Possibly, the shell provides all the calcium necessary for the egg layers, since Deroceras eggs are only partly calcified, and lack the characteristic outer shell of A.ater (Bayne, 1968b).

SELF-FERTILIZATION IN PULMONATES

The majority of pulmonates are simultaneous hermaphrodites. The ova and sperm are produced by a single gonad and the gametes traverse a common, hermaphrodite duct. It is possible, therefore, for oocytes to be fertilized by the animal's own sperm.

Self-fertilization produces offspring which are genetically like the parent. This is expedient if the environmental conditions remain constant since the characteristics which favoured the parent will now favour its progeny and result in a high level of survival (Selander and Kaufman, 1973). Cross-fertilization however produces greater genetic variation. Some of the offspring may be less well adapted to the existing conditions, but the variety of genotypes ensures that should conditions change, some individuals may still survive. Long-term survival of the species therefore favours cross-fertilization, but self-fertilization becomes advantageous when populations are depleted or widely dispersed.

The simultaneous hermaphrodites, therefore, might be expected to have some mechanism by which the foreign sperm preferentially fertilize the ova. This, however, presents difficulties since the animal's own sperm and ova come into contact long before any foreign sperm are

encountered, and cross-fertilization would be the exception rather than the rule.

There are several possible ways of overcoming this:

(i) the presence of foreign sperm in the reproductive tract may somehow inhibit the action of the animal's own sperm (Cain, 1955).

(ii) the sperm may be incapable of fertilizing the ova until they have passed along the reproductive tract or received the copious secretions associated with copulation (Thompson and Bebbington, 1969; Bayne 1973).

(iii) the ova may mature as they pass along the reproductive tract and are not ready to be fertilized by the large mass of sperm in the gonad or seminal vesicle (Ikeda, 1937; Horstmann, 1955; Bayne, 1973).

(iv) any combination of these.

If no foreign sperm are received over a long period however, it is advantageous if the restrictions on self-fertilization can be overcome.

While it is comparatively easy to demonstrate self-fertilization in isolated animals (Table 19) the relative occurrence of cross-and self-fertilization in normal breeding populations is more difficult to determine. Ikeda (1937) and Cain (1955) used genetic markers for body pigment and followed the inheritance of pigmentation in Philomycus bilineatus and Lymnaea stagnalis respectively.

In both species cross-fertilization was predominant immediately after copulation. In L.stagnalis this continued for approximately 100 days, but then there was an abrupt change to self-fertilization which Cain suggested was due to depletion of the foreign sperm. Although this sperm remains viable for over three months it is never passed onto a third snail at subsequent matings.

Similarly, P.bilineatus normally resorted to self-fertilization when the stocks of foreign sperm were exhausted. But this species revealed an additional complication. The authors claim these cross-breeding experiments indicate that the individual's own sperm, which had reached the bursa copulatrix, actually returned to the site of fertilization along with the foreign sperm. Consequently, some oocytes in the fertilization chamber were self-fertilized and some

Table 19 Self-fertilization reported in the Pulmonata

(i) Basommatophora

Oken	1817	Lymnaea auricularis
Colton	1918	Ancylus sp.
		Lymnaea spp.
		Physa sp.
		Planorbis spp.
Robson	1923	Paludestrina jenkinsi
Seshaiya	1927	Lymnaea luteola
Crabb	1928	Lymnaea pallustris
Colton and Pennypacker	1934	Lymnaea columella
Cain	1955	Lymnaea stagnalis
Monteiro et al.	1984	Biomphalaria spp.

(ii) Stylommatophora

Laurent	1851	Limax flavus
Wotten	1893	Arion ater
Lang	1904	Helix
Luther	1915	Agriolimax agrestis
Künkel	1916	Arion ater
		Limax cinereus
		Limax cinereoniger
		Limax variegatus
Künkel	1934	Limax tenellus
Ikeda and Emura	1934	Bradybaena similaris stimpsoni
Ikeda	1937	Philomycus bilineatus
Maury and Reygrobellet	1963	Deroceras agreste
		Deroceras leave
		Deroceras meridionale
Bayne	1973	Succinea grosvenori
		Succinea unicolor
Davies	1977	Arion intermedius

cross-fertilized, even though fresh, foreign sperm was readily available.

More recently, the inheritance of albinism has been studied in snails of the genus Biomphalaria (Monteiro et al., 1984) and this has revealed a third variation of self-fertilization. At copulation sperm is transferred from one animal to another. Some sperm will cross-fertilize the ova, but some is stored and passed onto a third individual at a subsequent mating. If this happens to be the original partner self-fertilization can occur and in small populations this would not be uncommon. In B.obstructa the sperm from more than one partner can be stored at a time and therefore the sperm of several individuals can be transferred at a single mating. Monteiro et al. have termed this phenomenon "sperm sharing."

The mechanism of self-fertilization in the Arionidae and Limacidae is completely unknown, and any of these methods for self-fertilization may occur in the wild. In the laboratory D.reticulatum rarely produces self-fertilized eggs (Runham and Hunter, 1970; Duncan, 1975) although other members of the genus will do so when kept in isolation (Luther, 1915; Maury and Reygrobellet, 1963). Arion hortensis on the other hand has never been mentioned in association with self-fertilization. This may simply be due to lack of

information, but may imply that this species, like D.reticulatum, avoids self-fertilization. Arion ater (Wotten, 1893, Künkel, 191b) and A.intermedius (Davies, 1977) have all bred successfully without mating.

To confirm the observations on D.reticulatum and to see if A.hortensis will produce self-fertilized eggs, members of both species were reared in isolation.

Materials and methods

Eggs produced by stock D.reticulatum and A. hortensis were incubated in the animal house and the newly-hatched animals were isolated after three weeks. At this age they are still immature (Gonad stage B) and have not started to copulate. Young slugs separated before three weeks rarely survive. In all other respects the animals were reared normally.

The incidence of egg-laying was recorded and each clutch of eggs was incubated to assess the level of fertility.

Results (See Table 20)

(i) D.reticulatum

Out of 38 D.reticulatum 26 laid eggs. Eight animals produced just single clutches, containing between 6 and 19 eggs, but they were all infertile. Eighteen animals laid regularly, producing, on average, 6 clutches during their reproductive life. Only five of these slugs, however, laid fertile eggs, with 80-100% hatching successfully (Table 21).

Egg-laying commenced when the slugs were 70-121 days old and generally continued for a further 4-5 months.

(ii) A.hortensis

Out of the 35 A.hortensis 26 laid eggs. Eight animals produced single clutches, of which 6 were infertile, containing between 2 and 12 eggs. The two fertile clutches were larger, with 23 and 29 eggs, and approximately 90% of these hatched. Eighteen animals laid regularly, producing, on average, five clutches during their reproductive life. Only one of these slugs consistantly laid infertile eggs. All the others self-fertilized and in each clutch over 89% hatched successfully. In some cases there were more hatchlings

Table 20 INCIDENCE OF SELF-FERTILIZATION

	EGG BATCHES PRODUCED				NO EGGS PRODUCED	TOTAL NO. OF ANIMALS
	REGULARLY		IRREGULARLY			
	FERTILE	INFERTILE	FERTILE	INFERTILE		
D.reticulatum	5	13	-	8	12	38
A.hortensis	17	1	2	6	9	35

Table 21 HATCHING DATA FOR REPRESENTATIVE ANIMALS PRODUCING SELF-FERTILIZED EGGS

	No. of eggs laid	No. of hatchlings	% hatch	Length of incubation in days
D.reticulatum	12	12	100	28
	21	20	95	28
	35	31	89	26
	30	30	100	31
	29	25	86	29
	14	13	93	33
A.hortensis	19	25	132	31
	32	32	100	28
	34	32	94	32
	28	25	89	33
	12	12	100	31

than eggs. When these batches were observed under the microscope two embryo slugs were frequently seen sharing a single egg.

Egg-laying commenced when the animals were 82-105 days old and then continued for a further six months.

Discussion

These results show that although both species are capable of fertilizing their own eggs, only A.hortensis regularly does so when reared in isolation. D. reticulatum seldom resorted to self-fertilization, but egg production was not normally suppressed and consequently, many animals laid infertile clutches. This suggests that ovulation is independant of mating, and will proceed as soon as the slugs become sexually mature.

In some basommatophoran species self-fertilization depends upon self-copulation (Colton, 1918; Noland and Carriker, 1946; Cain, 1955), the copulatory organ inserting the sperm package directly into the female duct. The anatomy of the Stylommatophora, however, precludes this, but if solitary individuals go through the motions of courtship and copulatory behaviour and then retain the sperm package, there is no reason why this sperm should not be treated as foreign sperm and

thus fertilize the ova. The factors which inhibited self-fertilization by the supply of stored sperm could still apply, since (i) there is a sperm package in the reproductive tract, (ii) the journey through the reproductive tract permits capacitation, and (iii) delayed maturation of the oocytes could still prevent self-fertilization in the gonad or hermaphrodite duct. Self-fertilization by the animal's own sperm package would not succeed, however, if there was some physiological requirement based on the recognition of foreign sperm or sperm mass material.

The precise mechanism of self-fertilization in D.reticulatum and A.hortensis is not known. These animals may retain their sperm packages, as described above, or, possibly, prolonged isolation somehow removes the inhibition of self-fertilization. It is impossible to assess this behaviour histologically, since the presence of sperm in the bursa copulatrix could result from the regular disposal of aging sperm and therefore gives no indication that a sperm package has been formed. Also, the small amount of sperm which avoids digestion in the bursa, is stored in the seminal vesicle, where it cannot be distinguished from the animal's own sperm.

In both species, the slugs which had a tendancy for self-fertilization, did so consistantly throughout their reproductive life, and there were no cases where fertile and infertile clutches were laid by the same animal. Why some slugs should successfully fertilize their own eggs when others do not remains a mystery.

The incidence of twins appears to be quite arbitrary. Since it has been observed in eggs which have been cross-fertilized, it does not seem to be a direct result of self-fertilization. Wolda (1967) reported that in Cepaea nemoralis twins hatched when eggs were treated with distilled water. The eggs of D.reticulatum and A.hortensis were incubated in petri-dishes kept damp with moistened cellulose wadding and excess distilled water could possibly account for the irregular appearance of twins in A.hortensis.

In this laboratory polyembryony has been observed in the newly-laid eggs of D.reticulatum (Runham, pers. comm.). Distilled water cannot have had an affect on embryo development at this stage, and it appears that total isolation of the oocytes is not always achieved before they receive the albumen layer. This is a more likely explanation for the twinning seen in A. hortensis.

GENERAL DISCUSSION AND SUMMARY

Certain questions regarding the phylogeny of the slugs arise from these morphological and behavioural results. Firstly, the talon, absent in the African slug, *Aillya*, is seen in its simplest, looped form in the Arionidae, Philomycidae and a single representative of the Limacidae, *Milax gagates*. The remaining limacid slugs have the basic stylommatophoran arrangement consisting of a flattened, ciliated pouch which wraps around a single, simple diverticulum. Elaboration of the pouch and/or diverticulum gives the variety of form seen throughout the order, reaching a peak of complexity in the Bulimulidae. Does the simplified form of carrefour seen in the arionid and, to a certain extent, the limacid slugs indicate a primitive or advanced state, and should *M.gagates* be reclassified according to its carrefour structure?

Secondly, the sperm package is seen in its simplest form in *D.reticulatum*. Does this represent the ancestral spermatophore or has the jelly mass evolved to parallel changes in the mode of sperm transfer?

There is a general evolutionary tendency towards the loss of the shell. For example, within the Zonitidae a progressive lightening of the shell can be seen in *Zonitoides*, *Retinella*, *Oxychilus* and *Vitrea*.

In the neighbouring family, Vitrinidae, the shell is extremely thin and may be overgrown by the mantle, while the Limacidae show the most reduced form. A parallel pattern of evolution can be seen in the Endodontacea, where the primitive endodontid pulmonates give rise to the Arionidae, and is repeated within the Bulimulidae, Urocyclidae and Helicarionidae, all three families having slug-like members.

The Endodontidae and Zonitidae represent the smallest and earliest stylommatophorans and their habitat is restricted by their dependence on calcium and water availability. Without the structural protection of a shell the arionid and limacid slugs have developed both physiological and behavioural adaptions raising their tolerance to water loss, and this, together with reduced calcium demands, increases their range of habitat.

Thus, there seems to be no question that the slug-like form is an advanced adaptation to different environmental pressures, but this does not imply that both carrefour and spermatophore structure have advanced simultaneously. Certainly, there is a basic stylommatophoran carrefour, and there may be an evolutionary tendency towards increased complexity of diverticula, but a direct line of evolution from looped to diverticulate carrefour (or vice versa) seems

unlikely, and the two forms probably arose independently, the former only persisting in the Endodontacea. This suggests that the members of the Endodontacea, including the Arionidae and Philomycidae, represent a branch of the evolutionary tree completely separate from other Stylommatophora. To confirm this the talon types of "related" species must be established.

Assuming that the talon is an accurate marker for systematic classification, M.gagates must be reviewed and its position amongst the Limacidae reconsidered.

It is impossible to determine whether the jelly mass of D.reticulatum is an advanced or primitive form of sperm package. The presence of prostate granules in the trifid appendage suggests homology with the flagella of spermatophore-producing snails, and the different type of granule indicates a potentially different function for this secretion. The advanced level of development shown by slugs, however, does perhaps favour the idea that this is a highly specialized, but simplified, form of the sperm package which may have evolved in parallel with the development of specific copulatory characteristics.

Summary of reproduction in D.reticulatum and A.hortensis

The environmental control of reproductive activity functions at two levels. Firstly, there is the long term response to photoperiod which controls the onset of maturation, and secondly, there are the short term factors of temperature, humidity, food availability etc. which have an immediate effect on egg laying.

In both D.reticulatum and A.hortensis short days retard growth and development so that winter hatchlings grow slowly and their food requirements are minimal. As spring approaches and the days lengthen the curb on maturation is lifted. This coincides with the new spring growth of vegetation, and so when energy demands are high, food is plentiful. Subsequent development is unaffected by the photoperiod and egg-laying commences as soon as the animals are fully mature. In addition, an endogenous rhythm appears to regulate reproductive activity and this may be responsible for synchronizing the breeding cycles.

The lengthy courtship procedures are made up of a series of repeated behavioural elements common to many species of slug. There is no simple chain of events and it seems probable that the individual sequences are integrated and controlled centrally by the nervous and/or endocrine systems. Courtship initiates the

release of sperm and stimulates secretion from the prostate gland. The formation of the spermatophore or jelly mass appears to take a finite length of time and subsequent delays in transferring the sperm package may arise from the two slugs failing to appose their genital atria, or possibly this extended period is necessary for the secretions to harden. If the latter case applies earlier copulation would result in incompletely formed packages.

The epiphallus and penial mass are functionally comparable. Here, the sperm package is formed and moulded into shape, and it is possible that both evert at copulation, the epiphallus 'degloving' to release the spined spermatophore and the penial sac fully everting to expose the jelly mass. The unprotected jelly-mass is very vulnerable at copulation. It is coated with a comparatively thin layer of prostate secretion and is exposed on the surface of the everted genital organs while being passed from one animal to the other. Its fusiform shape and flexible nature may therefore have arisen out of necessity since a stiff spermatophore would be unmanageable. In contrast, the more elaborate spermatophore of A.hortensis, with its hardened outer shell, must protect the sperm, and orientation of the surface spines allows easy transfer at copulation while discouraging withdrawal. In addition, it passes directly from the epiphallus to the entrance of the

bursa copulatrix and therefore avoids outside exposure. Such protection must favour successful sperm transfer.

Both sperm and jelly mass are placed into the entrance of the bursa copulatrix and remain in position after the copulating pair have separated. Peristalsis in the muscular walls of the bursa then pulls the sperm package into the digestive sac. Sperm may be released from the spermatophore via a pore in the tail or it may split along the ridge of spines and escape into the atrium. The jelly mass on the other hand needs no specialized exit pore or line of weakness, and simple pressure exerted by muscular contraction of the bursa would rupture the thin coating of secretion. There is no evidence to suggest that sperm which has entered the bursa, is subsequently released.

The sperm's progress towards the carrefour needs further investigation. The present results suggest that in D.reticulatum it passes along the male groove of the common duct, while in A.hortensis the female tract is implicated. This apparent disparity must be confirmed.

The finger-like carrefour diverticulum of D.reticulatum functions only temporarily as a spermatheca. The sperm is not stored in the carrefour and in both D.reticulatum and A.hortensis it passes into

the hermaphrodite duct where it joins the animal's own supply of sperm. This inevitably poses the question of how the small amount of foreign sperm preferentially fertilizes the ova. Observations on isolated animals suggest that self-fertilization is somehow suppressed, but the problem can only be resolved when a suitable genetic marker is found or radio-active labelling localizes sperm of different origins. It is interesting to note that in animals with more prominent or elaborately lobed central diverticula, this part of the carrefour acts as a spermatheca.

Throughout the pulmonates the hermaphrodite duct functions as a seminal vesicle, storing the animal's own sperm and that received at copulation, and the epithelial cells are adapted for propelling, immobilizing, phagocytosing and/or maintaining sperm.

The sperm may not fertilize the eggs immediately, but wait within the oocyte cytoplasm until both maturation divisions are complete. Only then do the sperm and egg nuclei fuse. Thus, the precise moment of fertilization may occur at any point along the reproductive tract. This suggests that the fertilization pouch is not necessarily an accurate term for the pouched diverticulum of D.reticulatum or the ascending limb of the carrefour loop in A.hortensis, it may merely control the flow of oocytes or ova, while

impluses transmitted via the extensive nerve plexus stimulate the albumen gland to release a fixed aliquot of secretion per egg.

The timing of egg-laying is regulated by changing environmental conditions, mediated by endocrine and/or nervous centres in the brain. This results in eggs being laid when conditions are optimal and maximizes the slug's reproductive potential.

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