

## Sensory innervation of the ovotestis in the snail *Helix aspersa*

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### Summary

Because oviposition in the land snail *Helix aspersa* is a metabolically expensive process coupled to a high fixed cost, one expects oviposition to occur only when the clutch size surpasses a minimum value at which the reproductive benefit exceeds the cost. We propose that neural innervation of the gonad allows *H. aspersa* to monitor oocyte production and ensure an adequate supply of gametes prior to ovulation. The ovotestis is innervated by a branch of the intestinal nerve in which the majority of axon fibres measure  $<0.2\ \mu\text{m}$  in diameter. We found a strong positive correlation between the number of mature oocytes in the ovotestis and the frequency of spontaneous afferent spikes in the nerve branch. Tactile stimulation of the ovotestis resulted in a 20-fold increase in afferent

spikes and an efferent reflex directed towards the ovotestis and the pericardium. Afferent activity also increased 10-fold after an experimentally induced increase in the volume of the ovotestis. These results suggest that the growing oocytes expand the walls of the acini and trigger action potentials in the mechanosensitive nerve terminals that lie within the acinar walls. We hypothesize that the resulting tonic signal is permissive for ovulation. In addition, a phasic sensory signal may occur during ovulation to trigger CNS motor output related to oviposition.

Key words: oviposition, clutch size, oocyte, ovotestis, sensory innervation, land snail, *Helix*.

### Introduction

Oviposition in land snails requires the successful completion of several metabolically costly processes, including oogenesis, albumen production, egg calcification and nest excavation (Tompa, 1984). Furthermore, oviposition is strongly influenced by environmental factors such as climate, photoperiod, competition and parasitism (Chase, 2002; Wayne, 2001). Taken together, these considerations suggest that egg laying is a resource-limited process that should occur relatively infrequently. Indeed, even under the optimized conditions of a breeding farm, only 13% of snails *Helix aspersa* oviposit more than once every month, while the majority (56%) oviposit no more frequently than once every three months (Daguzan, 1981). Clutch sizes in *H. aspersa* vary considerably between populations and under different conditions, with reported means ranging from 59 (Koene and Chase, 1998) to 108 (Daguzan, 1981).

Obviously, as clutch size increases, so too does the energetic cost to the female function. It is important to note, however, that while clutch sizes vary, each oviposition event is coupled to a fixed minimum cost. Factors contributing to the fixed cost of egg laying include the search for adequate soil conditions (travel distances of up to 15 m in *Helix pomatia*; Pollard, 1975), nest excavation and the generation of hydrostatic pressure to expel the egg mass (Perrot, 1938). Since the fixed costs are high, it is reasonable to attribute the infrequency of

oviposition to the fixed costs. The cost per egg can be reduced, however, by producing large clutches. Furthermore, as clutch size increases, so too does the offspring survival rate because large clutches are less prone to desiccation (Bayne, 1968). On the other hand, predation risks may favour more frequent depositions of smaller clutches at multiple sites. Ultimately, the number of eggs laid by an individual is determined by balancing energetic costs against reproductive benefits. We propose that oviposition events occur when the clutch size surpasses a minimum value for which the reproductive benefit exceeds the total of fixed and variable costs. Therefore, a prerequisite for egg laying should be the availability of the threshold number of mature oocytes. In the present study, we address the question of how *H. aspersa* is able to monitor its oocyte production to ensure the presence of this minimum number of gametes before it initiates the costly cascade of physiological events that results in oviposition.

In 1914, Schmalz described the innervation of the ovotestis (OT) in *H. pomatia* by a branch of the intestinal nerve. The functional significance of this innervation, however, remains unknown. Similarly, innervation of the mammalian ovary has been described in several species, including pigs (Majewski et al., 2002), rats (Burden and Lawrence, 1977) and humans (Hill, 1962), but information regarding the function of such innervation is sparse. Anatomical clues and lesion studies

suggest a sensory role possibly related to follicular recruitment (Aguado, 2002), steroidogenesis (Kawakami et al., 1981) or blood flow regulation (Ojeda and Lara, 1989). Here, we describe further details of the innervation of the ovotestis in *H. aspersa*, and we demonstrate a sensory function. We conclude that sensory endings in the gonad respond to the volumetric increase that occurs with oocyte maturation and that the neural signal is relayed to the central nervous system (CNS) via a low-frequency, tonic discharge of afferent spikes.

## Materials and methods

### Animals

Adult specimens (curled shell lips) of the land snail *Helix aspersa* Müller were obtained from Mary's Garden (Strathmore, CA, USA). Snails were kept in large Lucite boxes (36 cm×36 cm×8 cm) at 18–21°C under a reversed 16 h:8 h light:dark photoperiod. They were cleaned every 2–3 days and fed a diet of powdered grains and chicken feed supplemented with calcium.

### Morphology and innervation

The innervation of the gonad was examined after labelling the ovotestis branch of the intestinal nerve with neurobiotin (Vector Labs, Burlington, ON, Canada). The nerve branch was cut at its point of entry into the ovotestis. The distal nerve stump was sucked into a glass capillary that was then filled with 8% neurobiotin in 0.1 mol l<sup>-1</sup> Tris buffer (pH 7.6). Infiltration was allowed to continue for 24 h. After fixation in 4% paraformaldehyde, the preparation was incubated for 16 h in 4% Triton X-100 in 0.1 mol l<sup>-1</sup> phosphate-buffered saline (pH 7.4). For visualization, we used the Vectastain ABC kit (Vector Labs). Preparations were initially examined as wholemounts, then cut as 20 µm frozen sections.

For electron microscopy, a nerve segment was fixed in 5% glutaraldehyde in 0.1 mol l<sup>-1</sup> sodium cacodylate (pH 7.2) and dehydrated in acetone. Epon sections were stained with 4% uranyl acetate and Reynolds lead citrate (Reynolds, 1963). Photomicrographs were digitized, then analysed using Sigma Scan software (SPSS Inc., Chicago, IL, USA). The perimeter of every fibre profile was individually traced using a mouse-driven cursor. For each profile, we calculated the diameter (feret diameter) of a hypothetical circular object with an area equivalent to the measured profile.

### Electrophysiology

Nerve recordings were obtained from reduced preparations consisting of the CNS, the albumen gland, the seminal vesicle and the OT embedded in the digestive gland (Fig. 1). The entire preparation was pinned down in a Sylgard-coated recording dish comprising a CNS chamber, an OT chamber and a central gap (Fig. 1). The intestinal nerve was desheathed and passed through small slits in the Sylgard walls of the central gap that were subsequently sealed with Vaseline. During recordings, either saline or an isotonic sucrose solution was perfused through the central gap at a flow rate of approximately

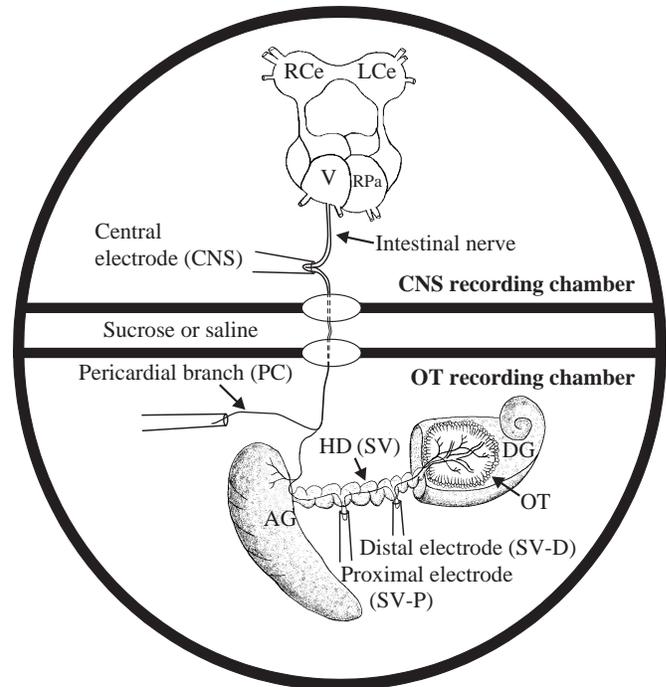


Fig. 1. Schematic drawing of the semi-intact preparation and perfusion dish. During some experiments, the gap in the centre of the dish was perfused with sucrose to reduce efferent traffic in the intestinal nerve; otherwise, all tissues were bathed in saline. AG, albumen gland; CNS, central nervous system; D, distal; DG, digestive gland; HD, hermaphroditic duct; LCe, left cerebral ganglion; OT, ovotestis; P, proximal; RCe, right cerebral ganglion; RPa, right parietal ganglion; SV, seminal vesicle; V, visceral ganglion.

10 ml min<sup>-1</sup>, while the recording chambers were always filled with snail saline (Kerkut and Meech, 1966).

To avoid possible artefacts associated with cut nerves, we recorded from the intact intestinal nerve using *en passant* glass suction electrodes. To assess whether recorded spikes were afferent or efferent, it was necessary to record simultaneously from at least two locations on the nerve (Fig. 1). In the OT recording chamber, one distal electrode and one proximal electrode (hereafter referred to as SV-D and SV-P, respectively) were placed on the nerve adjacent to the seminal vesicle portion of the hermaphroditic duct. There was no branching of the nerve between SV-D and SV-P, and nearly all recorded action potentials were detected at both electrode sites. A third central electrode was placed on the nerve in the CNS recording chamber (CNS electrode). In some experiments, a fourth electrode was placed in the OT chamber to monitor activity from the proximal stump of the pericardial branch of the intestinal nerve (PC electrode). The amplified signals were digitized using the Digidata 1322A converter and viewed using Axoscope 8.0 software (Axon Instruments, Union City, CA, USA).

A naive participant identified afferent spikes in the records. SV-D and SV-P traces were viewed simultaneously on a

computer screen with an expanded time scale. A moveable vertical line provided by Axoscope was used to assess the intervals between pairs of spikes when both members of the pair were judged to be from the same unit based on waveform similarities and inter-spike intervals. A spike that appeared first at SV-D and second at SV-P was identified as afferent, whereas a spike appearing first at SV-P followed by SV-D was identified as efferent (see Fig. 4B). Since the number of afferent spikes was much less than the number of efferent spikes, the latter was determined by subtraction. First, the total number of spikes with amplitudes exceeding 100% of the noise level was counted using Mini Analysis software (Synaptosoft, Decatur, IL, USA). Then, the number of identified afferent spikes was subtracted from the total to give the number of efferent spikes. To facilitate the identification of afferent spikes, most of the efferent activity was suppressed during recording periods devoted exclusively to assessing afferent discharges; this was accomplished by perfusing sucrose through the gap. The directional nature of spikes in the pericardial nerve branch was not considered because these recordings were obtained from the proximal nerve stump; Mini Analysis was used to obtain the total spike count.

#### *Tactile stimulation*

To test for sensory responses, a wooden applicator stick was held by a micro manipulator, and its cut end (2 mm diameter) was lowered onto the centre surface of the OT. The probe covered approximately 50% of the OT surface area. Responses were evoked when the probe was moved laterally across the surface of the OT using a knob on the manipulator. A back-and-forth movement was employed, the total duration of which was 3 s. Punctate stimuli of shorter duration were ineffective.

#### *Oocyte and egg counts*

After completing the electrophysiological recordings, fine forceps were used to break apart the connective tissue covering the OT. Suction from a 1 ml micropipette was used to collect the OT homogenate and transfer it to a test tube. By using coded tubes and examining homogenates in groups of two or more, the oocytes were counted without bias. Small volumes (1.4 ml) of the OT extract were successively transferred to a shallow glass dish and viewed under a dissecting microscope at 20× magnification. A grid system was used to ensure that each oocyte was counted only once. Each oocyte was measured on two perpendicular axes and the resulting mean was taken as the cell's diameter. Only mature oocytes at the vitellogenic stage (diameter >100 µm) were counted (Griffond and Bolzoni-Sungur, 1986). Sperm were not counted because they are stored in the seminal vesicle not the OT.

To ensure an adequate representation of snails with high oocyte counts, some individuals were selected after they exhibited nest-excavating behavior (a prelude to egg laying), since oviposition is performed only by snails with a large store of mature oocytes. To obtain nest-excavating snails (hereafter referred to as 'diggers'), a group of approximately 50 snails

was transferred to a Lucite box that contained 5–7 cm of moist potting soil and sand in a 1:1 mixture. Diggers were removed before they could oviposit, and they were used for electrophysiological experiments within 3–5 days.

Counts of oviposited eggs were obtained after placing snails on soil as described above. Once a snail had departed from its nest, the deposited eggs were removed, washed and counted.

## Results

### *Morphology of ovotestis innervation*

The major features of the OT, as illustrated in Fig. 2A, are the acini, in which the gametes develop, and the ductal system through which the gametes exit the gonad. The organ itself is embedded in the digestive gland, with only the top surface exposed after dissection. Oocytes appear dark in Fig. 2A and can be seen lying at the distal ends of the exposed acini. A fine branch of the intestinal nerve travels along the portion of the hermaphroditic duct known as the seminal vesicle and terminates within the OT, as originally described by Schmalz (1914). As the nerve extends distally along the duct, fibres branch laterally to innervate structures within the OT (Fig. 2B). These fine branches disperse throughout the organ, and some are visible within the acinar walls (Fig. 2C). In none of our five successful neurobiotin backfill preparations did we observe any peripheral cell bodies.

We examined the OT branch of the intestinal nerve in cross sections at the level of the seminal vesicle or just before it enters the OT. Here, the nerve measures only 20 µm in diameter but it contains 3025 fibre profiles (Fig. 3A). Two types of fibres appear to be partitioned in distinct regions of the nerve. The smaller of these regions is approximately 5 µm×5 µm, contains approximately 2250 fibre profiles and is surrounded by darkly stained glial processes (Fig. 3B). Nearly all of these profiles measure <0.25 µm in diameter. Larger fibres comprise the remaining 80% of the cross-sectional area. Because many of the larger profiles are irregularly shaped, the dimensions of all fibres were measured as feret diameters. Overall, 57% of the fibres have diameters of <0.20 µm; the largest diameters are 2.1 µm. It is likely, based on numerous studies in other animals, that the smaller fibres serve sensory functions, while the larger fibres have motor functions.

### *Afferent discharges*

To address a possible sensory role of OT innervation, we first examined the effect of OT stimulation on spike activity in the intestinal nerve. Using SV-D, SV-P and CNS electrodes (Fig. 1), we recorded a significant increase in spike frequency following tactile stimulation of the OT (Fig. 4A). Quantification of afferent spikes was performed on the basis of spike timing at SV-P and SV-D electrodes (Fig. 4B). Our reason for preferring this laborious procedure over the alternative of recording from a distal nerve stump is that early recordings of the latter type exhibited prominent bursting patterns and much greater spike rates than did recordings from an intact nerve. We concluded that the nerve lesion caused a

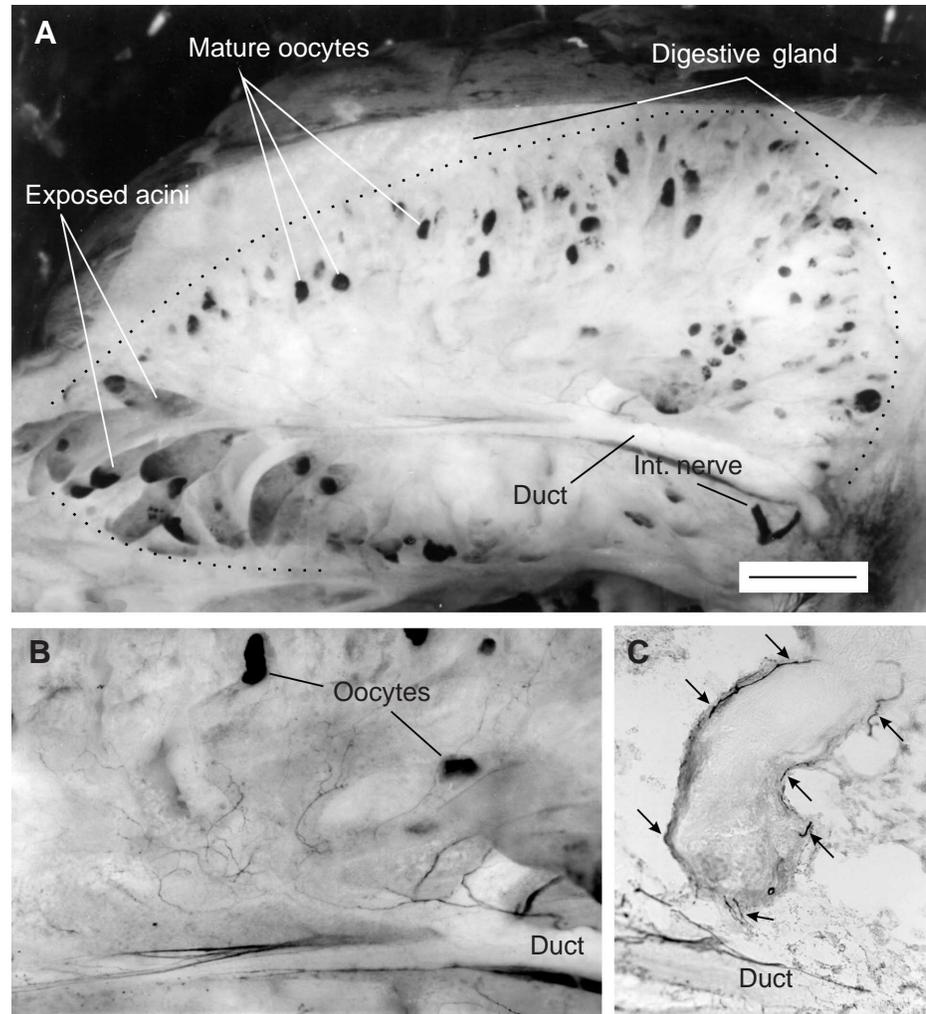


Fig. 2. Innervation of the ovotestis (OT) in *Helix aspersa*. (A) Wholemount of the OT embedded within the digestive gland. The dotted line delineates the perimeter of the OT. Neurobiotin was applied to the cut end of the intestinal nerve in this preparation (note the darkened stump), and the oocytes reacted nonspecifically to the histochemical procedure used to visualize the nerve. (B) Magnification of a portion of the hermaphroditic duct and OT from A showing fine branching in the intestinal nerve. (C) Frozen section (20 µm) from a second preparation showing innervation (arrows) along the wall of an acinus. Scale bars: A, 1 mm; B, 550 µm; C, 170 µm.

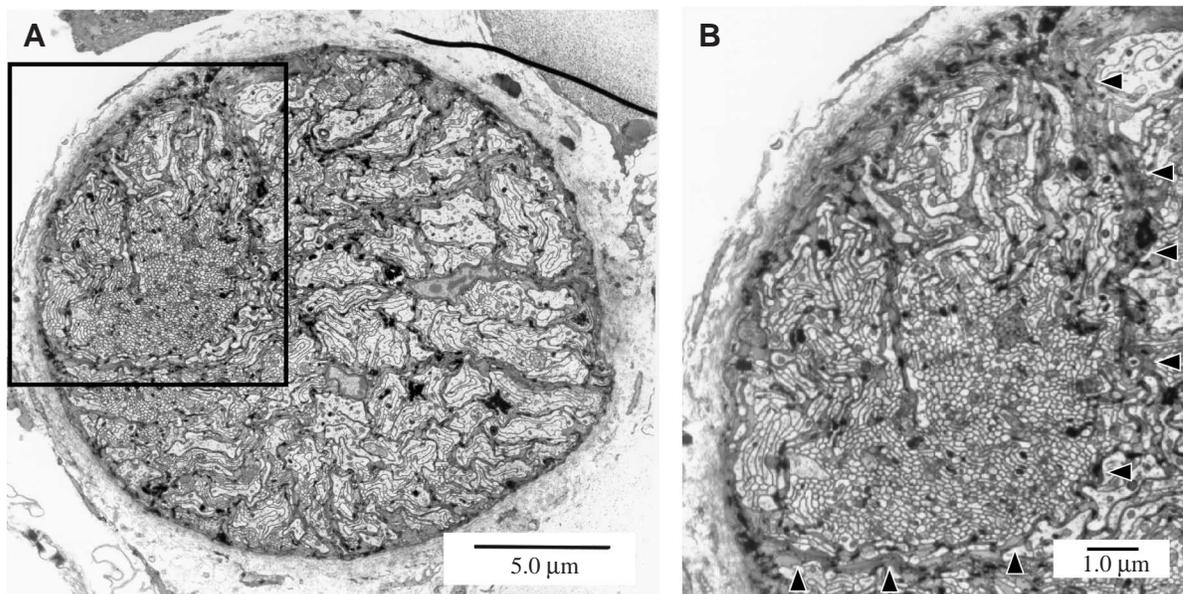


Fig. 3. Electron micrographs of the intestinal nerve in cross section. (A) Section of the nerve at the level of the seminal vesicle. There are 3025 fibre profiles, of which 1732 (57%) have diameters <0.20 µm. (B) Enlargement of the boxed area in A showing the region of very small diameter fibres. Note the surrounding boundary formed by processes of darkly staining glial cells (arrowheads).

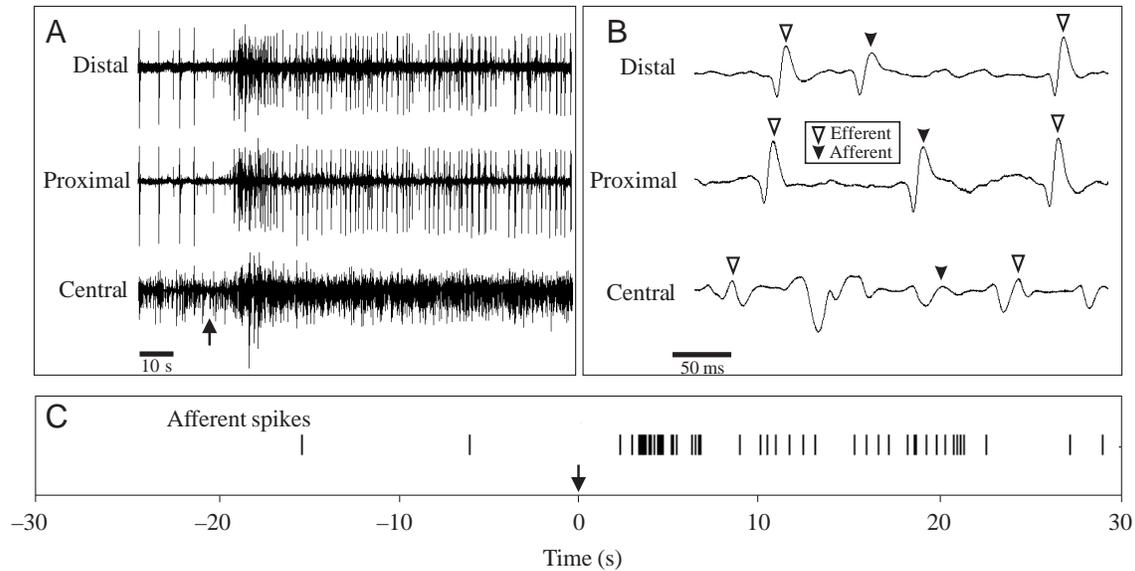


Fig. 4. Effect of ovotestis (OT) mechanical stimulation on the frequency of afferent spikes in the intestinal nerve. (A) Representative traces from three electrodes placed on the intact nerve (see Fig. 1). A wooden applicator stick mounted on a micro manipulator was used to gently stroke the surface of the OT for 3 s; arrow indicates the onset of stimulation. (B) A representative portion of the record from A is amplified to illustrate the discrimination of efferent spikes (open arrowheads) and afferent spikes (filled arrowheads) based on inter-spike intervals recorded at distal and proximal electrodes positioned along the seminal vesicle; signals recorded at the central electrode were not used for this purpose. (C) Raster plot showing the timing of afferent spike activity for 30 s periods before and after the stimulus. The arrow at time 0 indicates the onset of mechanical stimulation.

persistent increase in excitability, yielding anomalous results. With the intact nerve, OT stimulation resulted in a 20-fold increase in afferent spike frequency (Fig. 4C). Concomitantly, we observed a 40-fold increase in overall spike frequency at the CNS electrode. These results indicate a sensory function for the OT innervation.

To further elucidate the sensory role of innervation we examined spontaneous afferent spike rates in relation to the number of mature oocytes present in the OT. The frequency of afferent spikes varied from 1.0 spikes  $\text{min}^{-1}$  with 31 oocytes present to 79.2 spikes  $\text{min}^{-1}$  with 118 oocytes present. In a sample of 14 snails, the afferent spike rate was strongly correlated with the number of oocytes in the OT (Fig. 5A). A linear regression analysis on log-transformed data revealed a highly predictive relationship ( $r^2=0.812$ ,  $P<0.001$ ; Fig. 5B). This result suggests that information regarding oocyte number in the OT reaches the CNS *via* the intestinal nerve. Furthermore, we found a significant relationship between log afferent spike frequency and log efferent spike frequency ( $r^2=0.623$ ,  $P<0.001$ ; Fig. 5C), suggesting that afferent spikes originating in the OT reach the CNS and cause increased firing in neurons that project back to the OT. Fig. 5A,B shows that animals selected as nest diggers had significantly higher oocyte counts than did non-diggers. Diggers had a mean ( $\pm$ S.E.M.) oocyte count of  $116.2\pm 4.3$  while non-diggers had a mean oocyte count of  $53.8\pm 14.2$ . These numbers are in line with the  $86.9\pm 2.7$  fertilized eggs deposited during 104 oviposition events observed in our laboratory colony of snails. Notably, we observed a jump in the frequency of afferent activity when the

number of oocytes approached 87 (Fig. 5A). The mean afferent spike frequency in all animals with fewer than 87 oocytes was 4.23 spikes  $\text{min}^{-1}$ , whereas the mean frequency in animals with oocyte counts exceeding 87 was 44.5 spikes  $\text{min}^{-1}$ . Thus, the data suggest the presence of a threshold oocyte value below which afferent spike activity is low and above which spike frequency increases substantially.

#### Experimental inflation of the ovotestis

Because oocytes grow to large sizes (up to 250  $\mu\text{m}$ ), at which point their diameters exceed that of the acini and ducts within which they develop, it can be assumed that they will eventually exert pressure on the innervated walls of the acini and ducts. Therefore, to examine the mechanism of sensory excitation, we artificially inflated the OT to simulate the expected volumetric expansion ( $N=3$ ). First, we cut the hermaphroditic duct at the junction of the OT and the seminal vesicle. We then inserted a cannula into the distal portion of the duct and injected fast green dye (0.01 g per 100 ml saline) into the OT. For the experiment illustrated in Fig. 6, we used an animal that had very few (17) oocytes. Injections of 10  $\mu\text{l}$  or 20  $\mu\text{l}$  were made incrementally at 5 min intervals. As can be seen by the traces shown in Fig. 6A, the combined afferent and efferent activity increased significantly following the injections. When afferent spikes were analysed separately (raster display in Fig. 6A; Fig. 6B), the effect of OT inflation was even more apparent. While the afferent spike frequency prior to OT inflation was 4.5 spikes  $\text{min}^{-1}$ , this value increased 10-fold to 47 spikes  $\text{min}^{-1}$  after a total of 40  $\mu\text{l}$  saline had been

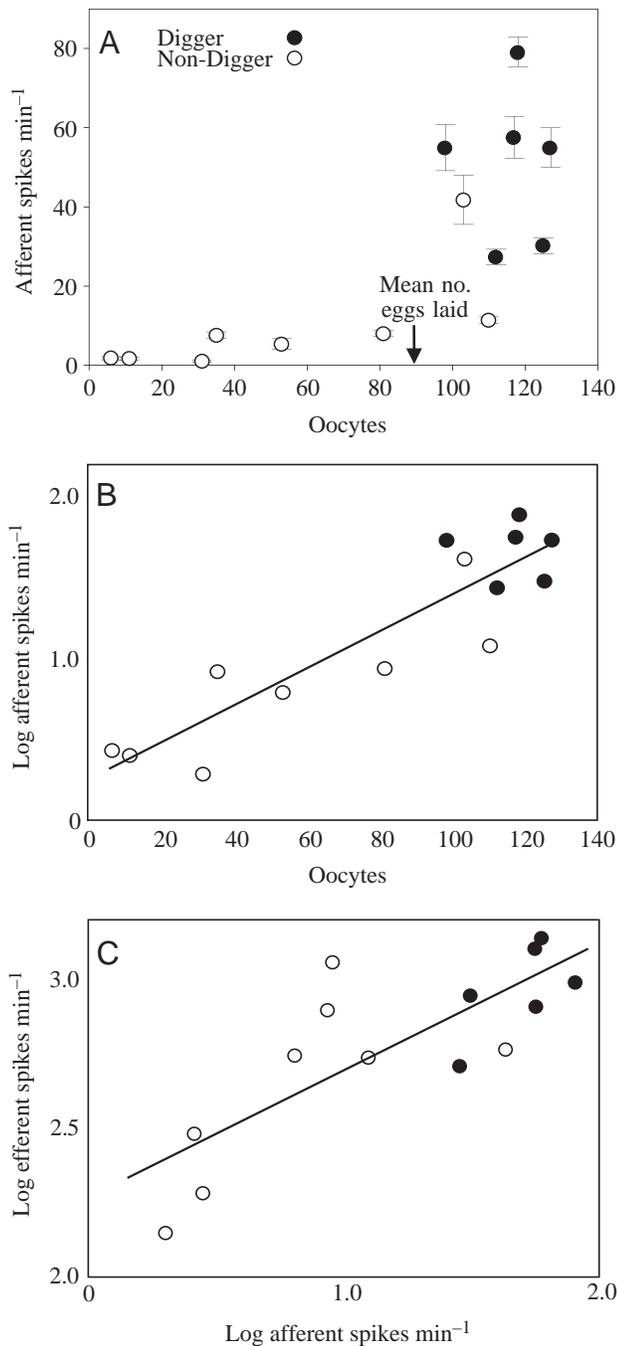


Fig. 5. Spiking activity in the intestinal nerve relative to the number of mature oocytes present in the ovotestis. (A) Recordings obtained from snails with large numbers of mature oocytes had afferent spike rates substantially higher than those recorded in snails with few mature oocytes. Data points are means  $\pm$  S.E.M. for five randomly selected periods of 1 min duration. Diggers were observed excavating nests within 5 days prior to the recordings. The number of deposited eggs was determined from our laboratory colony (mean =  $86.9 \pm 2.7$ ,  $N=104$ ). (B) Log transformation of the data shown in A. The line is the linear regression ( $r^2=0.812$ ,  $P<0.001$ ,  $N=14$ ). (C) Log-transformed data showing the relationship between afferent spike rates and efferent spike rates ( $r^2=0.623$ ,  $P<0.001$ ,  $N=14$ ).

injected. These data are consistent with the idea that the high levels of afferent spiking observed in animals with high oocyte counts are caused by a volumetric expansion of the OT associated with oocyte growth.

#### CNS efferent reflex

Given that the OT branch of the intestinal nerve responds to sensory stimulation at the periphery, we looked for an associated reflex output from the CNS. The intestinal nerve branches twice prior to its innervation of the OT, and the largest of these early branches innervates the pericardium (Schmalz, 1914). We recorded from the central stump of the severed pericardial branch while mechanically stimulating the surface of the ovotestis ( $N=6$ ). Fig. 7A shows that stimulation induced a surge in spike frequency detectable at the CNS, seminal vesicle and pericardial electrodes. In the OT branch, the afferent activity increased 4-fold after stimulation, while the concurrent efference increased 6.7-fold (Fig. 7B). In the central stump of the pericardial nerve branch, the spike rate increased 3.8-fold (Fig. 7B). These results demonstrate that OT sensory stimulation effectively elicits a reflexive response from the CNS. Moreover, it is noteworthy that the efferent response was sustained for more than 10 min following the brief stimulus.

#### Discussion

Knowledge that the ovotestis is innervated immediately raises questions of function, and our study has begun to provide some answers. Our results show that the innervation has at least a sensory function and probably a motor function as well. The most direct evidence of a sensory function is the finding that brief tactile stimulation of the organ causes a discharge of action potentials that is conducted centrally from a point near the nerve's insertion into the OT. However, the anatomical placement of the OT deep within the apical whorls of the hard shell makes it unlikely that the OT would ever experience mechanical stimulation *in vivo*. Rather, we propose that the natural stimulus for the nerve's sensory endings is the oocytes growing within the OT. Consistent with this idea is the strong correlation between afferent spike rates and the number of ripe oocytes. In fact, the log-linear relationship between these measures is in accord with the assumption that eggs are the natural stimulus. As we explain below, our results suggest that eggs generate at least two types of sensory signals for integration in the CNS.

#### Structure of the nerve

It is remarkable that the OT branch of the intestinal nerve, which measures only  $20 \mu\text{m}$  in diameter, should contain 3025 axon profiles. This is made possible by the inclusion of many fibres that approach the smallest size reported for any animal ( $0.05\text{--}0.1 \mu\text{m}$ ). Another intriguing aspect of the morphology is that the total number of profiles far exceeds that expected from the size of the nerve, in relation to the sizes and number of other nerves, and the number of neurons in the CNS. The central neuronal population in *Helix aspersa* is estimated to be

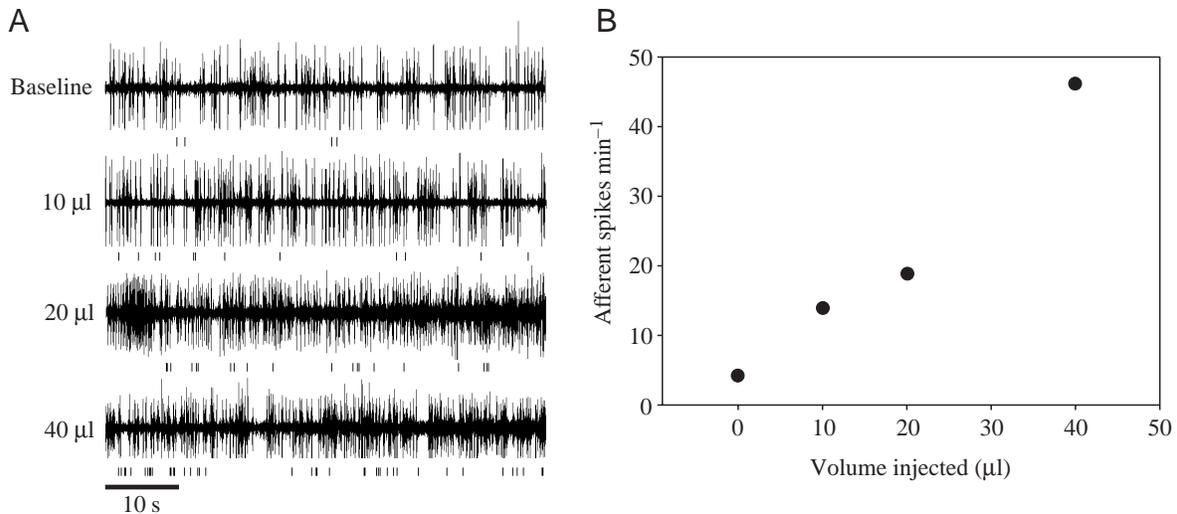


Fig. 6. Effect of ovotestis (OT) inflation on afferent spike frequencies in the intestinal nerve. (A) Representative recordings obtained 10 s after injecting saline at incremented levels of OT inflation. Below each trace is a raster plot showing the timing of afferent spikes. (B) Quantification of afferent spike frequencies from the traces shown in A.

~40 000 neurons (Chase, 2001; this value excludes the small olfactory interneurons in the procerebrum). Thus, as a first approximation, the number of axon profiles in the OT nerve is approximately 8% of the number of neurons in the CNS. However, because the circumference of the OT nerve is much less than 8% of the total circumference of all nerves combined, there is an apparent paradox. Possibly, the majority of the axons belong to peripheral sensory cells that, inexplicably, were not labeled when the OT nerve was back-filled with neurobiotin. Another possible explanation is provided by Pin and Gola (1984), who demonstrated, by Lucifer Yellow injections and novel electrophysiological experiments, that certain central neurons in *H. pomatia* send as many as 25 axons, in parallel, into the intestinal nerve. If this were generally true, then the 3025 axons in the OT would represent only 121 central neurons. However, this would raise the question, why do the axons branch so profusely before reaching their target organ?

#### A readiness signal

For the reasons presented in the Introduction, it would appear that *H. aspersa* delays laying eggs until it has a sufficient store of ripe oocytes. Our results demonstrate a close association between the number of ripe oocytes and the rate of spontaneous afferent discharge in the OT nerve branch (Fig. 5). Furthermore, there is a sharp increase in afferent activity when the oocyte count exceeds 87, which is the mean number of eggs actually laid by snails in our laboratory colony. We conclude that one function of the OT innervation is to signal the availability of a minimum quantity of ripe oocytes. Since oviposition requires that certain other conditions are also satisfied (e.g. soil composition and environmental moisture), we view the OT afferent activity as providing a permissive signal for oviposition, not a trigger.

The results from our OT inflation experiment (Fig. 6),

together with the anatomical data, suggest that the fine terminals of the OT nerve branch contain stretch-sensors capable of monitoring oocyte growth. The oocytes within the OT grow substantially as they mature from the early oogonia stage to the final vitellogenic stage, increasing 10-fold in diameter and 1000-fold in volume (Griffond and Bolzoni-Sungur, 1986). It is likely that this growth, which can occur concurrently in over 100 oocytes, results in a stretching of the acinar walls and the neuronal processes that lie within. From Fig. 2, it can be seen that the diameter of the largest oocytes (250 μm; Fig. 2A) is greater than the diameter of a typical acinus (~170 μm; Fig. 2C). However, the oocytes are nonetheless able to move in the acini and in the hermaphroditic duct because their shape is plastic. Similar examples of volumetric expansion causing sensory discharges in gastropod nerves have been reported for the anterior gut of *Aplysia* (Susswein and Kupfermann, 1975), the pro-oesophagus of *Lymnaea* (Elliott and Benjamin, 1989) and the prostate gland of *Lymnaea* (De Boer et al., 1997).

The frequency of afferent spikes is strongly associated with the frequency of efferent spikes conducted towards the OT (Fig. 5C), suggesting that, as the oocytes mature, the heightened afferent signal mediates the increased efferent signal. Here, we are discussing only the spike activity that occurs continuously and spontaneously; in the section below, we discuss the phasic responses following mechanical stimulation. If the efferent activity is continuous *in vivo*, it is probably not related to an imminent oviposition event. Rather, the efferent fibres might release a trophic factor that mediates the final stages of oocyte maturation. Or, the efference could provide a motor command to maintain tonus in the radial muscles surrounding the hermaphroditic duct, thereby retaining the oocytes until ovulation.

#### An ovulation signal

While the presence of mature eggs causes a tonic afferent

discharge in the OT branch of the intestinal nerve, tactile stimulation causes a phasic response (Figs 4, 7). Just as the tonic afferent discharge causes a tonic efferent discharge, the phasic afferent response causes a phasic efferent, or reflexive, discharge (Fig. 7). In both cases, the physical isolation of the CNS in our recording dish allows us to rule out mediation by diffuse chemical signals. We speculate that the natural stimulus for the phasic response is ovulation or, specifically, the passage of oocytes from their sites of attachment on the walls of the acini into distal segments of the hermaphroditic duct. We further propose that the sensory signal generated by the movement of

oocytes is responsible for orchestrating subsequent events in the multi-stage process of oviposition, in a manner similar to that proposed for the control of egg laying in *Aplysia* (Cobbs and Pinsker, 1982; Ter Maat and Ferguson, 1996).

Oviposition is a process that requires nearly 48 h of heightened metabolic activity (Perrot, 1938). A snail spends the majority of this time with its head beneath the soil surface in an extended, forward position. Thus, in addition to meeting the metabolic load, an ovipositing snail must increase its cardiac output to elevate its internal hydrostatic pressure. Cardiac output also plays an essential role in the mobilization

of calcium for eggshell calcification (Tompa, 1984). Just prior to oviposition, bound and unbound fractions of blood calcium increase by approximately 60% (Tompa and Wilbur, 1977). As in vertebrate and other molluscan species, the heart of *H. aspersa* is myogenic but subject to central nervous control (Chase, 2002). Therefore, to achieve the elevated heart rate necessary to accommodate the hydrostatic and metabolic demands of oviposition, CNS excitatory output to the heart is expected to increase. Consistent with this view, our experiments detected a dramatic acceleration of activity in the pericardial branch of the intestinal nerve following brief mechanical stimulation of the OT (Fig. 7). Also, electrical stimulation of the OT branch

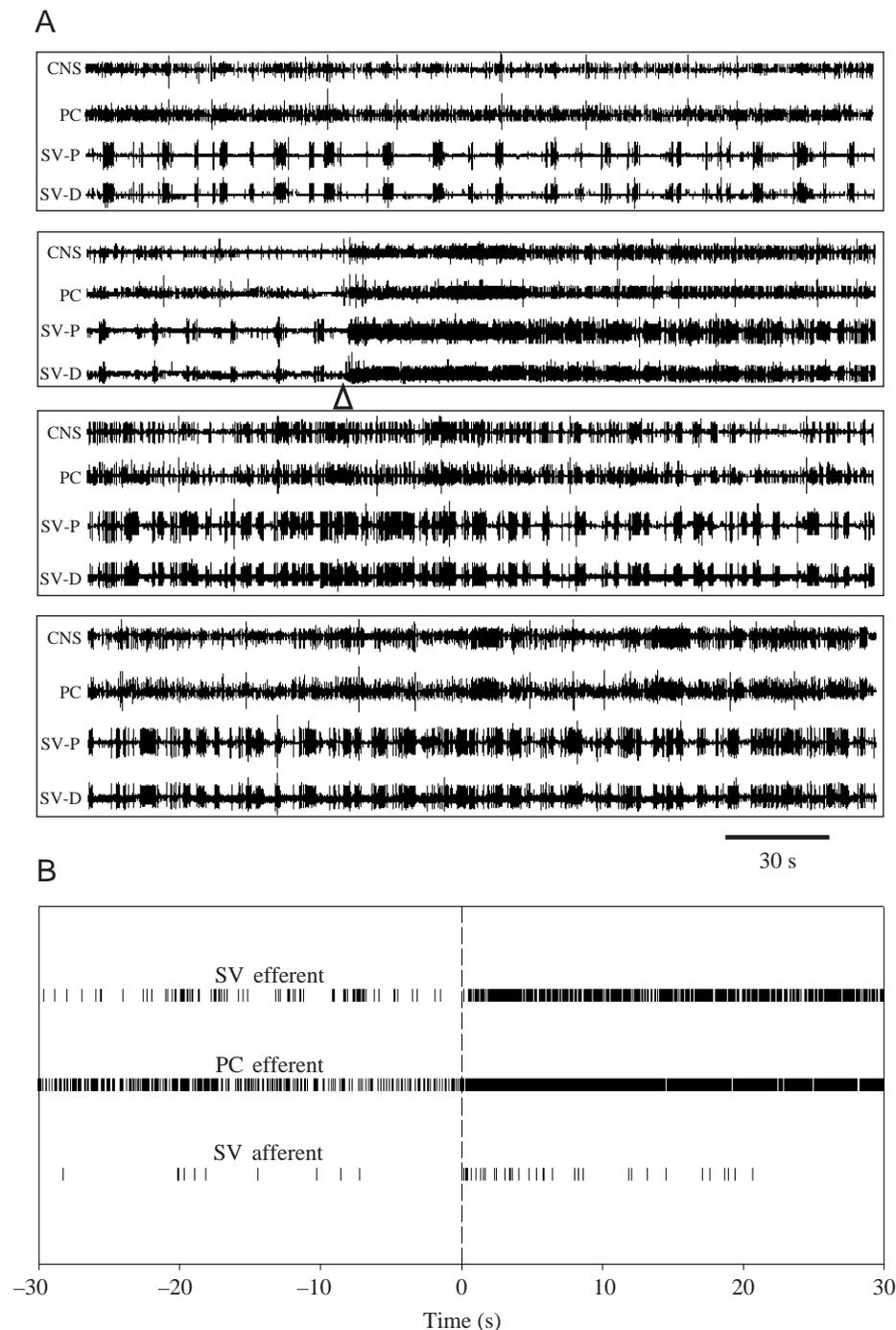


Fig. 7. Mechanical stimulation of the ovotestis induces an efferent reflex response from the CNS that travels into both the pericardial (PC) and ovotestis branches of the intestinal nerve. (A) Traces recorded at four positions on the intestinal nerve (see Fig. 1). Action potentials conducted to and from the ovotestis were recorded by two electrodes (SV-P and SV-D) placed on the nerve as it passes over the seminal vesicle; afferent vs efferent spikes were discriminated using timing differences at these two electrodes. The arrowhead indicates stimulation of the ovotestis with a wooden applicator stick (3 s duration). Note that an elevated level of activity is recorded at all electrodes in the 10 min period following the stimulus. (B) Raster plot showing the timing of afferent and efferent spikes recorded at seminal vesicle electrodes as well as efferent activity recorded at the pericardial electrode. Time 0 indicates the onset of tactile stimulation.

of the intestinal nerve produces a robust increase in heart beat amplitude (D. Weatherill, unpublished).

The OT of *H. aspersa* is another target of the efferent reflex elicited by mechanical stimulation. Studies in marine bivalves indicate that gonadal innervation is implicated in the maturation of oocytes and the induction of ovulation (Matsutani and Nomura, 1987; Ram et al., 1996). Studies in our laboratory are attempting to elucidate the role of efferent activity in the OT nerve branch of *Helix*. Thus far, neurobiotin labelling has revealed dense, varicose innervation of the distal hermaphroditic duct. Also, results indicate that efferent spikes, elicited by stimulation of the intestinal nerve, increase cilia beat frequencies and cause radial contractions in the hermaphroditic duct (E. Geoffroy, unpublished). These effects are consistent with a motor role for OT innervation in promoting the movement of oocytes from the OT to the fertilization chamber during the early stages of egg laying.

As we briefly summarized in the Introduction, there exists a substantial body of literature describing innervation of the mammalian ovary (reviewed in Aguado, 2002). A handful of such reports has characterized the innervation as sensory on the basis of anatomical clues, immunohistochemical results and lesion studies (Kummer et al., 1990; Majewski, 2002; Price and Mudge, 1983). However, to our knowledge, the results presented here constitute the first physiological evidence for sensory innervation of the gonad in any species. The sensory innervation of the OT in *H. aspersa* may provide insights into the function of analogous innervations in humans and other mammalian species.

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## References

- Aguado, L. I.** (2002). Role of the central and peripheral nervous system in the ovarian function. *Microsc. Res. Tech.* **59**, 462-473.
- Bayne, C.** (1968). Histochemical studies on the egg capsules of eight gastropod molluscs. *Proc. Malacol. Soc. Lond.* **38**, 199-212.
- Burden, H. W. and Lawrence, I. E., Jr** (1977). The effect of denervation on compensatory ovarian hypertrophy. *Neuroendocrinology* **23**, 368-378.
- Chase, R.** (2001). Sensory organs and the nervous system. In *The Biology of Terrestrial Molluscs* (ed. G. M. Barker), pp. 170-213. New York: CABI Publishing.
- Chase, R.** (2002). *Behavior and its Neural Control in Gastropod Molluscs*. New York: Oxford University Press.
- Cobbs, J. S. and Pinsker, H. M.** (1982). Role of bag cells in egg deposition of *Aplysia brasiliana*. II. Contribution of the egg movement to elicited behaviors. *J. Comp. Physiol. A* **147**, 527-546.
- Daguzan, J.** (1981). Contribution à l'élevage de l'escargot Petit-Gris: *Helix aspersa* Müller. *Ann. Zootech.* **30**, 249-272.
- De Boer, P. A. C. M., Jansen, R. F., Koene, J. M. and ter Maat, A.** (1997). Nervous control of male sexual drive in the hermaphroditic snail *Lymnaea stagnalis*. *J. Exp. Biol.* **200**, 941-951.
- Elliott, C. J. H. and Benjamin, P. R.** (1989). Esophageal mechanoreceptors in the feeding system of the pond snail *Lymnaea stagnalis*. *J. Neurophysiol.* **61**, 727-736.
- Griffond, B. and Bolzoni-Sungur, D.** (1986). Stages of oogenesis in the snail, *Helix aspersa*: cytological, cytochemical and ultrastructural studies. *Reprod. Nut. Dev.* **26**, 461-474.
- Hill, R. T.** (1962). Paradoxical effects of ovarian secretions. In *The Ovary*, vol. 2 (ed. S. Zuckerman), pp. 231-261. New York: Academic Press.
- Kawakami, M., Kubo, K., Uemura, T., Nagase, M. and Hayashy, R.** (1981). Involvement of ovarian innervation on steroid secretion. *Endocrinology* **109**, 136-145.
- Kerkut, G. A. and Meech, R. W.** (1966). The internal chloride concentration of H and D cells in the snail brain. *Comp Biochem. Physiol.* **19**, 819-832.
- Koene, J. M. and Chase, R.** (1998). Changes in the reproductive system of the snail *Helix aspersa* caused by mucus from the love dart. *J. Exp. Biol.* **201**, 2313-2319.
- Kummer, W., Gibbins, I. L., Stefan, P. and Kapoor, V.** (1990). Catecholamines and catecholamine-synthesizing enzymes in guinea-pig sensory ganglia. *Cell Tissue Res.* **261**, 595-606.
- Majewski, M., Kalczyk, J., Wasowicz, K., Bossowska, A., Gonkowski, S. and Klimaschewski, L.** (2002). Characterization of afferent and efferent galanin-containing nerve fibers in the porcine ovary. *Folia Histochem. Cytochem.* **40**, 261-268.
- Matsutani, T. and Nomura, T.** (1987). *In vitro* effects of serotonin and prostaglandins on release of eggs from the ovary of the scallop, *Patinoptecten yessoensis*. *Gen. Comp. Endocrinol.* **67**, 111-118.
- Ojeda, S. R. and Lara, H. E.** (1989). Role of the sympathetic nervous system in the regulation of ovarian function. In *The Menstrual Cycle and its Disorders* (ed. K. M. Pirke, W. Wuttke and U. Schweiger), pp. 26-32. Berlin: Springer-Verlag.
- Perrot, J.** (1938). La confection du nid e la ponte chez l'*Helix pomatia*. *Rev. Suisse Zool.* **45**, 221-235.
- Pin, T. and Gola, M.** (1984). Axonal mapping of the neurosecretory *Helix* bursting cells. Functional aspects of peripheral multibranching axons. *Comp Biochem. Physiol. A* **79**, 611-620.
- Pollard, E.** (1975). Aspects of the ecology of *Helix pomatia*. *J. Anim. Ecol.* **44**, 305-329.
- Price, J. and Mudge, A. W.** (1983). A subpopulation of rat dorsal ganglion neurons is catecholaminergic. *Nature* **301**, 241-243.
- Ram, J. L., Fong, P. P. and Kyoizuka, K.** (1996). Serotonergic mechanisms mediating spawning and oocyte maturation in the zebra mussel, *Dreissena polymorpha*. *Invert. Reprod. Dev.* **30**, 29-37.
- Reynolds, E. S.** (1963). The use of lead citrate at high pH as an electron opaque stain. *J. Cell Biol.* **17**, 208.
- Schmalz, E.** (1914). Zur Morphologie des Nervensystems von *Helix pomatia* L. *Z. Wiss. Zool.* **111**, 506-568.
- Susswein, A. J. and Kupfermann, I.** (1975). Localization of bulk stimuli underlying satiation in *Aplysia*. *J. Comp. Physiol.* **101**, 309-328.
- Ter Maat, A. and Ferguson, G. P.** (1996). Neuronal input contributes to sequence of *Aplysia* egg laying behaviors. *J. Comp. Physiol. A* **179**, 775-783.
- Tompa, A. S.** (1984). Land snails (Stylommatophora). In *The Mollusca*, vol. 7, *Reproduction* (ed. A. S. Tompa, N. H. Verdonk and J. A. M. van den Biggelaar), pp. 47-140. London: Academic Press.
- Tompa, A. S. and Wilbur, K.** (1977). Calcium mobilization during reproduction in the snail *H. aspersa*. *Nature* **270**, 53-54.
- Wayne, N. L.** (2001). Regulation of seasonal reproduction in mollusks. *J. Biol. Rhythms* **16**, 391-402.