

NERVOUS CONTROL OF MALE SEXUAL DRIVE IN THE HERMAPHRODITIC SNAIL *LYMNAEA STAGNALIS*

P. A. C. M. DE BOER, R. F. JANSEN, J. M. KOENE AND A. TER MAAT*

Graduate School Neurosciences Amsterdam, Research Institute Neurosciences Vrije Universiteit, Faculty of Biology,
De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

Accepted 2 January 1997

Summary

We studied the role of the prostate gland in determining the level of male sexual drive in the hermaphroditic pond snail *Lymnaea stagnalis*. Male sexual drive is high after a period of social isolation and decreases after copulation as a male. A positive correlation exists between the level of male sexual drive and the volume of the prostate gland. Like male sexual drive, the volume of the prostate gland increases during a period of social isolation and decreases after copulation as a male. Behavioural experiments demonstrated that animals with a lesion of the nerve that innervates the prostate gland (NP1) have a lower level of male sexual drive after social isolation than control animals. However, lesion of NP1 did not affect the increase

in the volume of the prostate gland caused by social isolation. Extracellular recordings from NP1 in a semi-intact preparation show a change in firing pattern during an experimentally induced increase in prostate gland volume. The results indicate that NP1 serves as a nervous pathway for the male sexual drive. We propose a simple motivational model for male sexual behaviour in *L. stagnalis* in which the volume of the prostate gland sets the level of male sexual drive.

Key words: copulation, motivation, reproduction, penis nerve, prostate gland, hermaphrodite, snail, *Lymnaea stagnalis*.

Introduction

The internal or motivational state of an animal is a factor that can cause variability in its behavioural patterns (Tinbergen, 1951). The effect of motivational factors on the execution of behavioural patterns has been widely investigated. Examples of such studies are those on feeding behaviour (insects: Hammer *et al.* 1994; mammals: Wilson *et al.* 1995; molluscs: Davis *et al.* 1977; Kupfermann, 1974; Kupfermann *et al.* 1991; Tuersley, 1989) and copulation behaviour (mammals: De Cantazaro and Gorzalka, 1980; Melis and Argiolas, 1995; molluscs: Adamo and Chase, 1990; Van Duivenboden and Ter Maat, 1985; Ziv *et al.* 1989). These studies have increased our understanding of behavioural variability. With this knowledge and the availability of neurophysiological research techniques, it is now possible to study the mechanisms that cause changes in the motivational state of the animal. The hermaphroditic pond snail *Lymnaea stagnalis* has provided a convenient model system for the study of motivational aspects of behaviour (Tuersley, 1989; Van Duivenboden and Ter Maat, 1985) and for the study of reproductive physiology (De Boer *et al.* 1996; Geraerts and Joosse, 1984; Hermann *et al.* 1994). Here, we investigate a mechanism underlying the motivational aspects of male sexual behaviour in *L. stagnalis*.

As Tinbergen (1951) pointed out, motivational factors are

responsible for the activation of a drive. Here, we define the tendency to copulate as a male as male sexual drive. The simultaneous hermaphroditic snail *L. stagnalis* copulates either in the male or the female role. Male sexual drive in *L. stagnalis* increases when copulation is prevented by social isolation. This increase in male sexual drive can be determined when animals are paired at the end of an isolation period: in pairs of snails that are composed of animals that have been isolated for different periods, those that have been isolated for the longest period become the male during copulation (Van Duivenboden and Ter Maat, 1985). Because the snails are always receptive, the level of male sexual drive sets the level of total copulatory activity. This makes male sexual drive an important aspect of the regulation of sexual behaviour.

Social isolation also stimulates copulation in other hermaphrodites, such as the molluscs *Helix aspersa* (Adamo and Chase, 1990) and *Aplysia fasciata* (Ziv *et al.* 1989) and the flatworm *Dugesia polychroa* (Peters *et al.* 1996). In these animals, isolation affects the total copulatory activity, i.e. both male and female. Susswein *et al.* (1993) suggested that the total copulatory activity in *Aplysia* spp. is also primarily directed by the level of male sexual drive. The mechanism through which social isolation induces a change in male sexual drive is not yet known. In *L. stagnalis*, the ejaculate consists of sperm cells

*Author for correspondence.

and secretory material, and these are stored in the seminal vesicles and the prostate gland, respectively (De Jong-Brink, 1984; Geraerts and Joosse, 1984). In this study, we explore the possibility that the amount of stored ejaculate could be a factor that induces changes in male sexual drive. We examined whether storage of ejaculate is affected by social isolation and whether information about this storage influences the male sexual drive.

Materials and methods

Animals

Adult specimens of *Lymnaea stagnalis* (L.) (shell heights 25–35 mm) were used. They were raised and kept in tanks under standard laboratory conditions with running fresh water (20 °C) under a 12 h:12 h light:dark cycle (Van der Steen *et al.* 1969). Lettuce leaves were provided *ad libitum*.

Mass measurements

The mass of the prostate gland and seminal vesicles was measured in group-accommodated and isolated snails. Animals from the first batch (G) were kept in groups for 8 days. Animals from the isolated batches were socially isolated by keeping them individually in perforated jars in the tank for 8 consecutive days. This period is sufficient to induce a significant increase in the male sexual drive (Van Duivenboden and Ter Maat, 1985). The animals from the second batch were only isolated for 8 days (I). Following the isolation period, the animals from the third and fourth batches were placed in groups, for either 1 (IG1) or 2 (IG2) days, during which time they were free to copulate. All animals were tested on the same day.

Fig. 1 shows the male reproductive tract of *Lymnaea stagnalis* and indicates which parts were dissected out. The prostate gland was removed by cutting the sperm duct (SD) and vas deferens (VD) close to the prostate gland (PG). The seminal vesicles (SV) are attached to the hermaphroditic duct (HD). Therefore, the part of the hermaphroditic duct with the seminal vesicles attached was dissected out. The proportion of hermaphroditic duct tissue was small compared with the amount of tissue from the vesicles. Therefore, the total dissected tissue will be referred to as the seminal vesicles. After dissection of the prostate gland and seminal vesicles, both tissues as well as the remains of the animal were weighed, dried in a Speedvac (Savant, Farmingdale, USA) and weighed again. During the dissection and weighing procedure, the investigator did not know what treatment each snail had undergone.

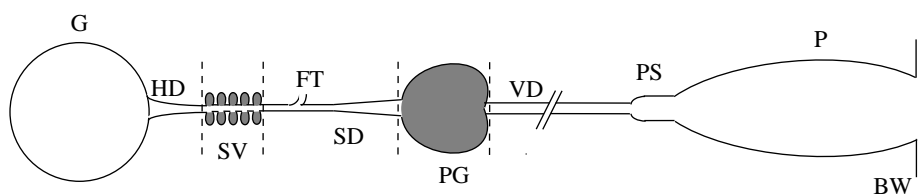
Tract tracing

The peripheral projections of the three nerve branches of the penis nerve, NP1, NP2 and NP3, were visualized using the tracer Neurobiotin. The method used is modified after Li and Chase (1995). Animals were dissected in 0.1 mol l⁻¹ phosphate buffer at pH 7.4. The preparations included the central nervous system (CNS), the penis nerve, the preputium, the prostate gland and the vas deferens. After identification of the three nerve branches, the surrounding tissue of one of the three was removed. The branch was cut and the peripheral end was sucked up into a glass micropipette. The pipette was then refilled with 8% Neurobiotin (Vector Laboratories, Burlingame, CA, USA) in 0.1 mol l⁻¹ Tris-HCl buffer (pH 7.4). The preparation was left at room temperature for 18–20 h, after which the tissue was fixed in 4% formaldehyde for 1 h. After several rinses with phosphate buffer, the preparation was incubated for 2 h with 4% Triton X-100 in phosphate buffer. The tissue was then incubated for 2 h in 5 ml of 4% Triton X-100 in phosphate buffer with one drop of solution A and one drop of solution B from the Vectastain ABC standard kit (Vector Laboratories). After several rinses with phosphate buffer, the tissue was incubated in 0.05% diaminobenzidine in phosphate buffer for 5 min before a drop of 30% hydrogen peroxide was added. The reaction was stopped after approximately 5 min by rinsing with phosphate buffer. After dehydration through an ascending alcohol series, the tissue was cleared in methyl salicylate and mounted in Entellan (Merck). The stained nerve branch was examined under a stereomicroscope.

Behavioural observations

We examined male copulatory behaviour in animals that were socially isolated as described above. Following 8 days of isolation, male sexual behaviour was measured as follows. Pairs of animals were placed in unperforated jars which were filled with water from the breeding tank and placed in a temperature-controlled room (20 ± 1 °C). The animals were observed for 3 h, during which time the different behavioural elements were quantified using software for the acquisition of behavioural data (The Observer, Noldus, Wageningen, The Netherlands). Following the experiment, female copulants were dissected and checked for the presence of semen in the vagina to determine whether transfer of semen had taken place. This is easily seen by the white swollen appearance of the vagina following transfer; the vagina is flat and difficult to recognize when no semen has been received. During the experiments, the observer did not know what treatment each snail had undergone.

Fig. 1. The male reproductive tract of *Lymnaea stagnalis*. Broken lines represent the locations of the cuts that were made to dissect the seminal vesicles (SV) and the prostate gland (PG). BW, body wall; FT, female tract; G, gonad; HD, hermaphroditic duct; P, preputium; PS, penis sheath; SD, sperm duct; VD, vas deferens.



Surgery

NP1 was lesioned in animals anaesthetised using 50 mmol l⁻¹ MgCl₂. An incision (2–3 mm) in the skin was made in the head region on the dorsal side just to the right of the median. After NP1 had been positively identified in the body cavity, either the nerve was cut or the animal was used for the sham-operated group. The operations were performed 8 days before the experiments. At the end of the experiments, the animals were killed to check whether the operations had been performed correctly.

Nerve recordings in a semi-intact preparation

Recordings were made from preparations consisting of the penis nerve, penial complex, prostate gland, vas deferens and part of the skin. For recordings from the intact NP1, the CNS was also included. The preparation was pinned down in a recording chamber lined with silicone elastomer. The recording chamber was filled with saline (pH 7.8) of the following composition (in mol l⁻¹): 4.0 CaCl₂, 1.7 KCl, 1.5 MgCl₂, 30.0 NaCl, 5.0 NaHCO₃, 10.0 NaCH₃SO₄ and 10.0 Hepes. The extracellular electrical activity of the peripheral cut end of NP1 was recorded using a glass microelectrode cut off at the appropriate diameter. An *en passant* electrode was used for recording the electrical activity of the intact NP1. This type of electrode was made from fine stainless-steel wire and is as described by Hermann *et al.* (1994). During the recordings, we mimicked the increase in volume of the prostate gland that normally occurs during social isolation. This was done by injection of saline at a constant rate of 1 µl min⁻¹ via a cannula in the sperm duct (SD, see Fig. 1). A pump (Minipuls 3, Gilson, Villieres le Bell, France) was used to achieve a constant rate of injection of saline.

The extracellular signals were amplified and filtered using a WPI DAM 80 AC differential amplifier (amplification 1000×, low-pass 1 kHz, high-pass 10 Hz) and stored on tape. The electrical recordings from the intact NP1 were digitized using a Cambridge Electronic Design A/D converter (model 1401, 12-bit). The recordings were analyzed by means of a template-matching algorithm to separate unitary waveforms (Jansen and Ter Maat, 1992). A group of waveforms that is defined with this method is referred to as one unit. Before statistical analysis, the individual spike train of each unit was reconstructed.

Statistical analyses

The minimum criterion for statistical significance was $P < 0.05$. We evaluated the mass data using analysis of covariance with the total body mass as a covariate. This was performed in order to eliminate some of the variance in the mass of the organs caused by the variance in total body mass. Although the animals were the same length and age, there was variation in their total body mass. This analysis was followed by a pair-wise comparison between the experimental groups (Bonferonni-adjusted). In the behavioural experiments, the data were subjected to a χ^2 -test for contingency tables. To evaluate differences between the interspike intervals in the

whole-nerve recordings and in the spike trains of the individual units, the Kruskal–Wallis test was used. This procedure is justified because no strong autocorrelations, of over 0.4, were detected (up to order 10) in the spike trains. To ensure that the autocorrelation structure of the spike trains would not influence the test statistics, we used only spike trains with more than 100 occurrences overall.

Group means are presented with their standard errors. Sokal and Rolf's (1995) description of statistical methods was used as reference for the correct statistical tests. The various tests were performed using Systat software (Evanston, USA).

Results

Effect of isolation and subsequent grouping on the mass of the prostate gland and seminal vesicles

We tested the effect of social isolation *versus* group-accommodation on the mass of the seminal vesicles and the prostate gland. Sperm cells and secretory material are stored in these organs (De Jong-Brink, 1984; Geraerts and Joosse, 1984). Four groups of animals were tested ($N=20$ for all groups): animals that were kept in groups (G), animals that were socially isolated (I) and animals that had been socially isolated and then placed in groups for 1 (IG1) or 2 (IG2) days, during which time they were free to copulate. As a control, we also measured the effect of the treatments on the total body mass. Before weighing, the animals that had been allowed to copulate were checked for copulatory activity.

The animals that had been allowed to copulate did show copulation behaviour in the tank. The animals were checked for copulatory behaviour twice a day with an 8 h interval (a successful copulation sequence takes one to several hours to complete; Van Duivenboden and Ter Maat, 1988). On the first day of grouping, all the animals were involved in copulatory activity at both inspection times. Half of the animals in both groups showed male copulation behaviour. On the second day, half of the IG2 animals were involved in copulatory activity and 25% were copulating in the male role. This observation was true for both inspections on day 2. Rudolph (1979) demonstrated that 95% of isolated snails that are allowed to copulate show reciprocation. Thus, 95% of the animals copulating in the female role adopted the male role at the end of copulation and *vice versa*. In the present experiment, it was found that after group housing all the snails had ejaculate in their female tracts and bursae, indicating that all had copulated as females. These data together with the behavioural observations make it reasonable to assume that the IG1 and IG2 snails in this experiment had copulated at least once as a male during group housing. The IG2 animals may have copulated even more times as a male.

The results of the weighing experiment are shown in Fig. 2. The dry mass of the prostate gland was significantly higher in I animals than in the other animals ($P < 0.001$). There was no difference between the G, IG1 and IG2 animals. The effects of the treatments on the mass of the prostate gland could not be due to a gross effect on the total body mass since this did not

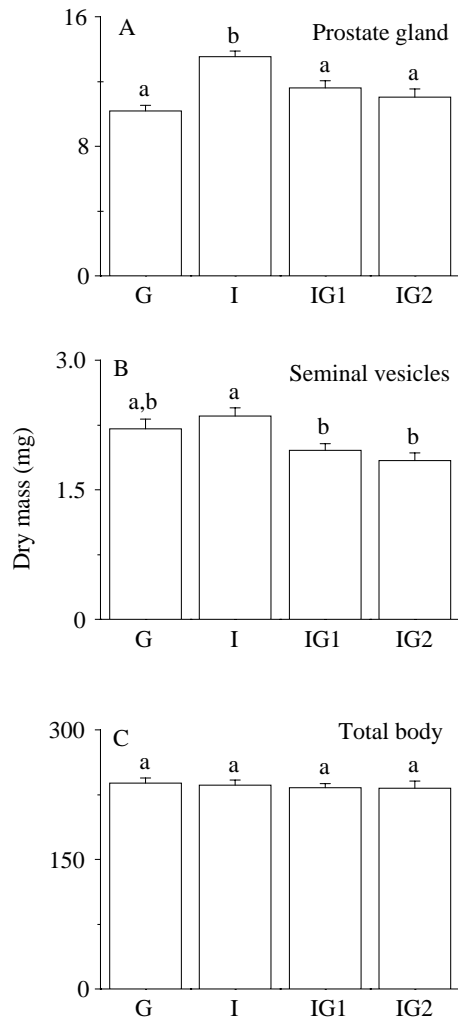


Fig. 2. Effect of social isolation and group-accommodation on the dry mass of the prostate gland (A), seminal vesicles (B) and the whole snail (C). Mean dry mass (+S.E.M.) is presented. The animals were continuously group-accommodated (G), socially isolated for 8 days (I), or group-accommodated for 1 (IG1) or 2 (IG2) days following an 8 day period of isolation. For all groups, $N=20$. Values for groups with different letters are statistically different ($P<0.05$).

change significantly (Fig. 2C). We therefore conclude that a period of 8 days of isolation induces an increase in the mass of the prostate gland. This effect is abolished when the snails are free to copulate again.

The dry mass of the seminal vesicles was not significantly different between the I and G animals (Fig. 2B). The I animals had a significantly higher seminal vesicle dry mass than the IG1 ($P<0.02$) and IG2 ($P<0.001$) animals. There was no significant difference between the dry mass of the seminal vesicles of the G, IG1 and IG2 animals. Thus, a period of isolation does not affect the mass of the seminal vesicles. There is, however, a significant decrease in mass when snails are allowed to copulate following a period of social isolation.

Because the ejaculate is an aqueous substance, a change in the mass of the seminal vesicles and the prostate gland implies

a change in the volume of these organs. For this reason, we will refer to the mass change of these organs as a volume change. Social isolation induces an increase in the volume of the prostate gland but not in the volume of the seminal vesicles; the volume of both organs decreases when snails are allowed to copulate after social isolation. The volume of the prostate gland and the level of male sexual drive are therefore correlated. It is possible that the volume of the prostate gland sets the level of the male sexual drive. This could be achieved either hormonally or neuronally. The latter possibility is investigated below.

Peripheral projections of the penis nerve

We examined whether a nervous connection exists between the prostate gland and the CNS. The peripheral projections of the penis nerve were investigated. The penis nerve innervates the preputium and penis (Elo, 1938). This nerve emerges from the cerebral ganglion on the right side of the CNS and projects to the preputium alongside the penis artery. Near the preputium, the penis nerve splits into three distinct branches termed NP1, NP2 and NP3. Anterograde fills were made of these three branches. These preparations showed that NP2 and NP3 project to the preputium and penis sheath, respectively. This observation is in agreement with earlier descriptions (Elo, 1938). However, the staining of NP1 revealed a previously unknown projection of this nerve which is reconstructed in Fig. 3. The nerve branch projects towards the base of the preputium (P) and continues on the dorsal side of the preputium towards the body wall on the right side of the animal, at the site where the anterior vas deferens (AVD) emerges from the body wall (BW). Approximately 5 mm more posteriorly, the posterior vas deferens (PVD), which originates at the prostate gland (PG), enters the body wall. The nerve branch runs along the surface of this piece of vas deferens. Near the origin of the vas deferens, NP1 divides into a large number of fine branches extending over the outside surface of the prostate gland.

These results show that NP1 provides a nervous connection between the prostate gland and the CNS.

Effects of NP1 lesion on the male sexual drive

To test whether NP1 is necessary for the increase in male sexual drive following isolation, we examined the effect of a lesion of NP1 on copulatory behaviour. Unoperated controls and a sham-operated group were included. All animals ($N=48$ for each group) were isolated for 8 days prior to the experiment. Normal male copulatory behaviour consists of six appetitive elements: mounting, circling, positioning, partial eversion of the preputium, total eversion of the preputium and probing. Copulation is completed with the consummatory element intromission (for a description, see De Boer *et al.* 1996). Probing always occurs during total eversion. Therefore, the number of animals showing total eversion equals the number of animals showing probing.

The number of animals showing the different behavioural elements of male copulation was significantly reduced

($P < 0.0005$ for all elements) in the group of lesioned animals (L) compared with unoperated (NO) and sham-operated (SO) animals (Fig. 4). Only three of the 48 animals in the L group showed the complete copulation sequence, whereas in the NO and SO groups this number was 18 and 13, respectively. There was no difference between the numbers of SO and the NO snails that completed copulation. Examination of the animals after the experiments showed NP1 had been cut successfully in all the animals that underwent a lesion operation, whereas all SO snails had an intact NP1. The results demonstrate that an intact NP1 is necessary to achieve the level of male sexual drive caused by 8 days of social isolation.

The above findings suggest that information about the prostate gland volume reaches the CNS *via* NP1. It is possible, however, that the lesion of NP1 simply prevented the increase in prostate gland volume from occurring and thereby prevented the increase in male sexual drive. Therefore, we examined the effect of a lesion of NP1 on the increase in prostate gland volume during 8 days of isolation. The dry mass of the prostate glands of isolated NP1-lesioned (IL) animals was compared with that of isolated unoperated (IN) and isolated sham-

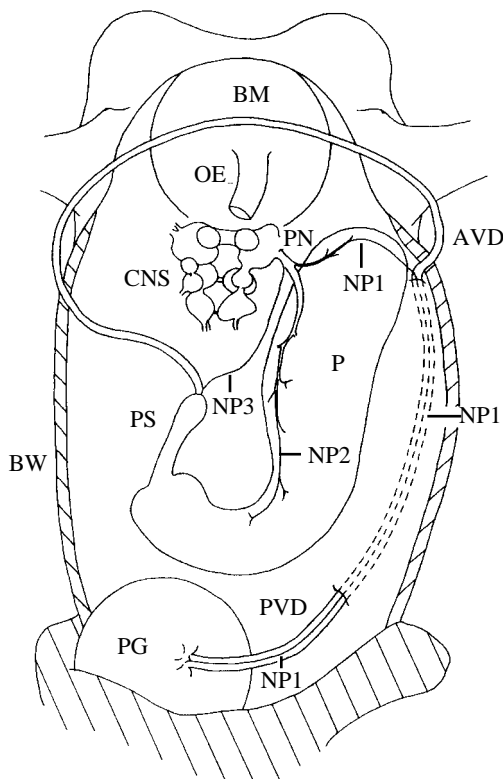


Fig. 3. The body cavity of *Lymnaea stagnalis* showing the projections of the three branches of the penis nerve, NP1, NP2 and NP3. Dorsal view of head/foot with the dorsal body wall and oesophagus removed. Broken lines represent the vas deferens and portions of NP1 that are located in the body wall. AVD, anterior vas deferens; BM, buccal mass; BW, body wall; CNS, central nervous system; P, preputium; PG, prostate gland; PN, penis nerve; PS, penis sheath; PVD, posterior vas deferens; OE, oesophagus.

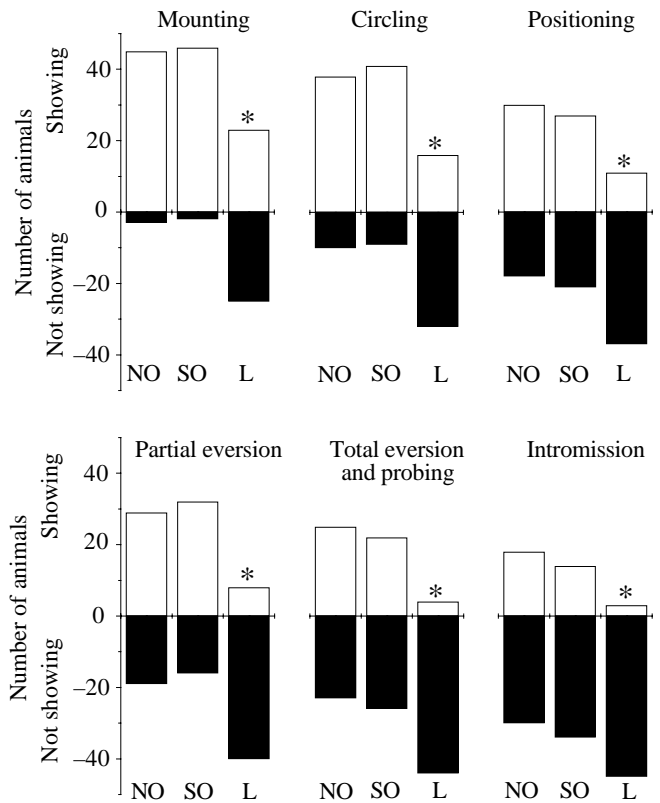


Fig. 4. Effect of a lesion of nerve NP1 on the number of animals showing (open columns) or not showing (filled columns) the elements of male sexual behaviour. All animals were socially isolated for 8 days prior to the behavioural analysis. The animals were put in pairs for 3 h for behavioural analysis. The number of animals showing the different behavioural elements of male copulation was significantly reduced in the lesioned animals (L) compared with unoperated (NO) and sham-operated (SO) animals; $*P < 0.0005$ for all elements. For all groups, $N = 48$.

operated (IS) snails and with group-accommodated, unoperated (GN) snails ($N = 16$ for all groups). All groups consisting of isolated snails, irrespective of whether they had a lesion of NP1, showed an increased mass of the prostate gland compared with GN snails ($P < 0.001$, Fig. 5). There was no significant difference in prostate gland mass among the isolated snails, including the IL snails. The treatment had no effect on the total body mass. This means that a lesion of NP1 does not prevent the increase in the volume of the prostate gland during 8 days of isolation, but that the effects of NP1 lesion are central.

Nerve end recordings

To examine whether electrical activity in NP1 contains information about the volume of the prostate gland, we artificially inflated the prostate gland while recording from the peripheral cut end of NP1. A semi-intact preparation consisting of a vas deferens, prostate gland and NP1 of a group-accommodated snail was used. The increase in volume of the prostate gland that occurs during isolation was mimicked by injection of saline. After 20 min of baseline recording, the

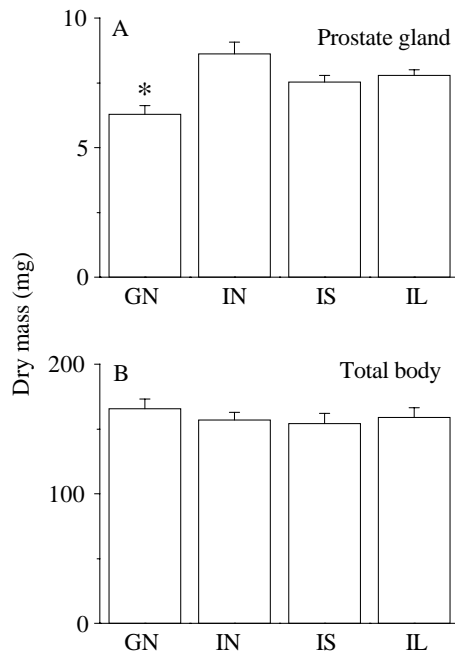


Fig. 5. Effect of a lesion of nerve NP1 on the dry mass of the prostate gland (A) after social isolation. Mean dry mass (\pm S.E.M.) of the prostate gland and total body mass (B) of socially isolated animals that had a lesion of NP1 (IL), that were sham-operated (IS) or that were unoperated (IN) and of group-accommodated animals that were unoperated (GN). There was no significant effect of the lesion on the mass of the prostate gland. There was a significant ($P < 0.001$) effect of social isolation on prostate gland mass. Lesion of NP1 does not affect the mass increase of the prostate gland that occurs as a result of isolation. For all groups, $N = 16$. The mean dry body mass of the animals was 158.9 ± 3.6 mg.

prostate gland was gently filled with saline for another 20 min period at a constant rate of $1 \mu\text{l min}^{-1}$ yielding a total increase of $20 \mu\text{l}$, which corresponds with the increase in wet mass of 20 mg that occurs after a period of 8 days of isolation (Fig. 2). Recordings were made from four animals. Fig. 6A shows the extracellular activity of NP1 during one representative recording. Soon (9.25 min) after the onset of injection, there was an increase in firing activity. This is shown in the plot of the cumulative number of spikes (Fig. 6B). This plot demonstrates that the firing activity in NP1 at the beginning of the recording is rather stable, as indicated by the linear rise in the cumulative number of spikes. However, approximately 9 min after the onset of the volume increase, this slope increases (arrowhead, Fig. 6B), indicating that the spiking activity has increased. The spike frequency before saline injection into the prostate gland began was 5.85 ± 0.9 spikes min^{-1} (mean \pm S.E.M.); after the start of the injection, it was 41.8 ± 2.4 spikes min^{-1} .

Not only the number of spikes but also the amplitude distribution of the spikes changed. Fig. 6C shows an overlay plot of two amplitude histograms. These data show that, during saline injection into the prostate gland, spikes with a larger amplitude appear in the signal recorded from NP1 and that

there is an increase in overall firing activity. Since the nerve from which we recorded was cut, we conclude that afferents project in NP1 and that these afferents respond to an increase in prostate gland volume with an increase in firing activity.

Whole-nerve recordings

The cut-nerve recordings indicated that a number of elements that project in NP1 respond to an increase in volume of the prostate gland. However, the recordings do not reveal whether all elements respond and whether their responses are similar. This information is needed to identify the elements that are responsible for the change in electrical activity that was measured in NP1 and thus the change in male sexual drive. For this purpose, spike train analysis was performed on whole-nerve recordings from the intact NP1 to investigate the effect of the increase in prostate gland volume on the different elements in NP1. It was found that action potentials measured from cut nerves are often distorted. This may be due to the physiological damage caused by the cutting of the nerve (R. F. Jansen, personal observation). Since stable action potentials are necessary for unit analysis, an intact NP1 was used to record the effect of volume increase in the absence of broken connections. A semi-intact preparation consisting of an intact NP1, the vas deferens, CNS and prostate gland of group-accommodated snails ($N = 7$) was used. The extracellular activity of NP1 was recorded using an *en passant* electrode. The volume of the prostate gland was increased using saline injection as described above. Fig. 7A (lower trace) shows the digitized compound nerve signal of NP1 during one representative recording. Ten minutes after the onset of injection, there was an increase in firing activity. This increase in overall spike activity occurred in all preparations between 5 and 10 min after the onset of injection (Table 1). The average interspike interval of these recordings decreased significantly after the onset of injection ($P < 0.025$, Table 1) in all recordings. During the control experiments, in which the prostate gland was not inflated ($N = 2$), no increase in firing activity occurred (Fig. 7A, top trace). In one recording, no significant change was found. In the other recording, there was a significant decrease in firing activity ($P < 0.025$, Table 1).

Units were separated on the basis of their waveforms by means of a template-matching algorithm. In the recording shown in the lower trace of Fig. 7A, 45 units were identified. The individual firing activities of the different units were reconstructed and are represented in Fig. 7B. A significant change in average interspike interval ($P < 0.05$) was found in 36 of the 45 units during filling of the prostate gland. All these 36 units showed a decrease in the average interspike interval. Two types of response could be identified. Examples of these two responses are given in Fig. 7C, which shows the cumulative number of spikes for two units during the recording period. The first type of response was found in 17 units and is illustrated in Fig. 7C by unit 33. Units of this type showed a periodic burst in firing activity visible as steplike increase in the cumulative spike count. The first large step occurred 10 min

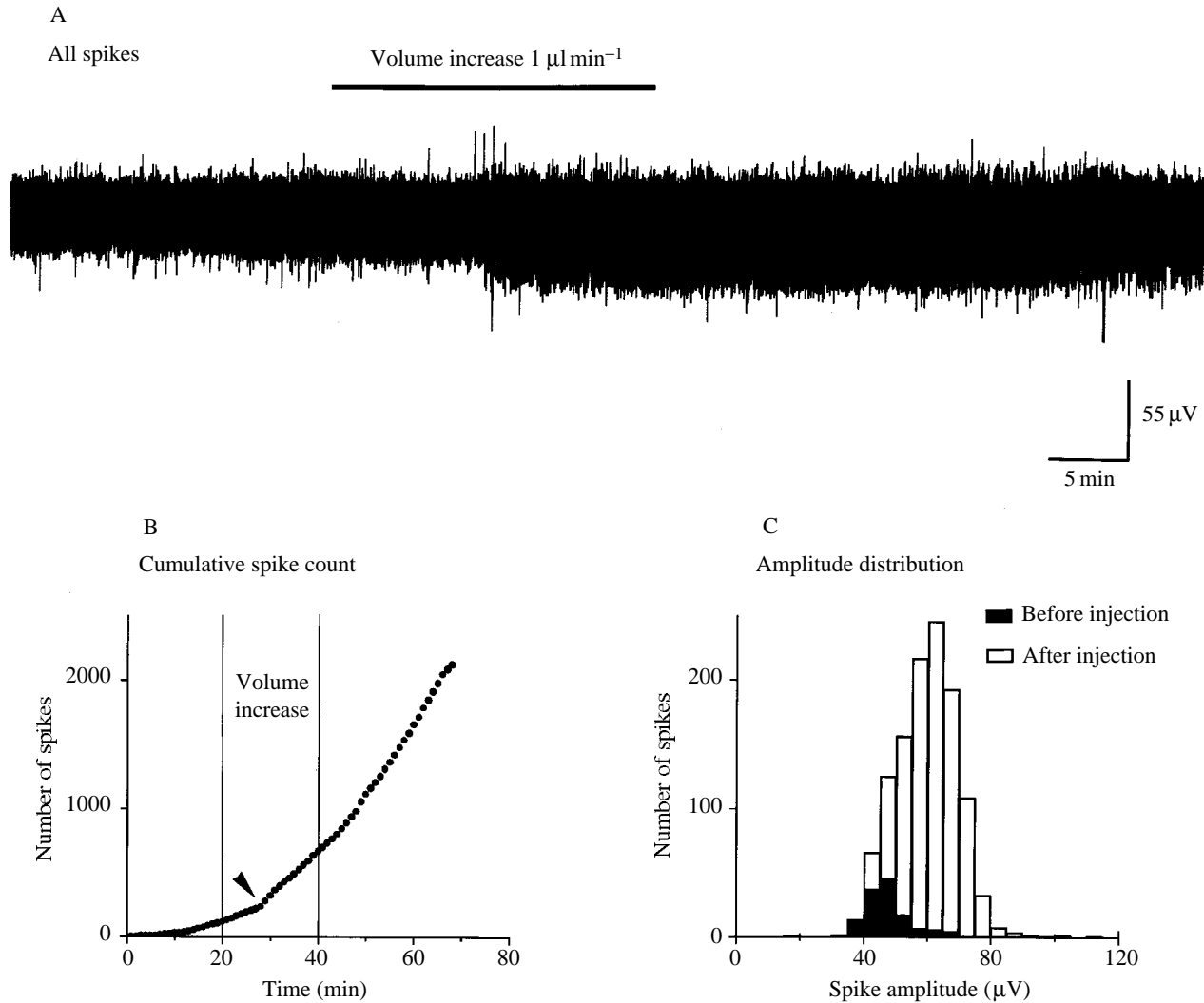


Fig. 6. Electrical recording from the peripheral end of nerve NP1 during an increase in the volume of the prostate gland induced by saline injection in a semi-intact preparation. (A) A recording from NP1 showing an increase in firing activity during and after the volume increase. (B) Cumulative spike count during the period of recording. Vertical bars mark the injection period. A linear slope for the number of spikes *versus* time data implies continuous firing activity. There is a significant increase in firing activity ($P < 0.001$) at 29 min (which is 9 min after the onset of the injection). This is visible as a change in slope (indicated with an arrowhead). (C) Overlay plot of two amplitude distributions. The filled bars represent the distribution during the 20 min prior to saline injection. The open bars represent the distribution during the 20 min after the end of saline injection. In the 35–40 μV bin, both bars have the same value.

after the onset of the volume increase. During the recording, 3–4 of these bursts occurred. The second type of response consisted of a lasting increase in firing activity. This was shown by 19 units and is illustrated in Fig. 7C by unit 42. The initial increase was detected approximately 10 min after the onset of the volume increase. This increase was either followed by a gradual increase in firing activity or by a steplike increase, such as in unit 33. The results show that the vast majority of the units in NP1 show an increased firing rate during and after injection of saline into the prostate gland. Table 1 shows the results from all seven replicate experiments. They all yielded the same outcome, and all the recordings contained the two categories of response. Analysis of the separate units from the control experiments showed that they did not have the response

pattern seen during inflation of the prostate gland (results not shown).

Discussion

In the present study, we examined the causes of the increase in male sexual drive that occurs during social isolation in the hermaphroditic snail *Lymnaea stagnalis*. It was found that an increase in male sexual drive after 8 days of isolation is positively correlated with an increase in prostate gland volume. Using the tracer Neurobiotin, it was shown that a nervous pathway (nerve NP1) exists between the prostate gland and the CNS. In snails with a lesion of this nerve, the level of male sexual drive following social

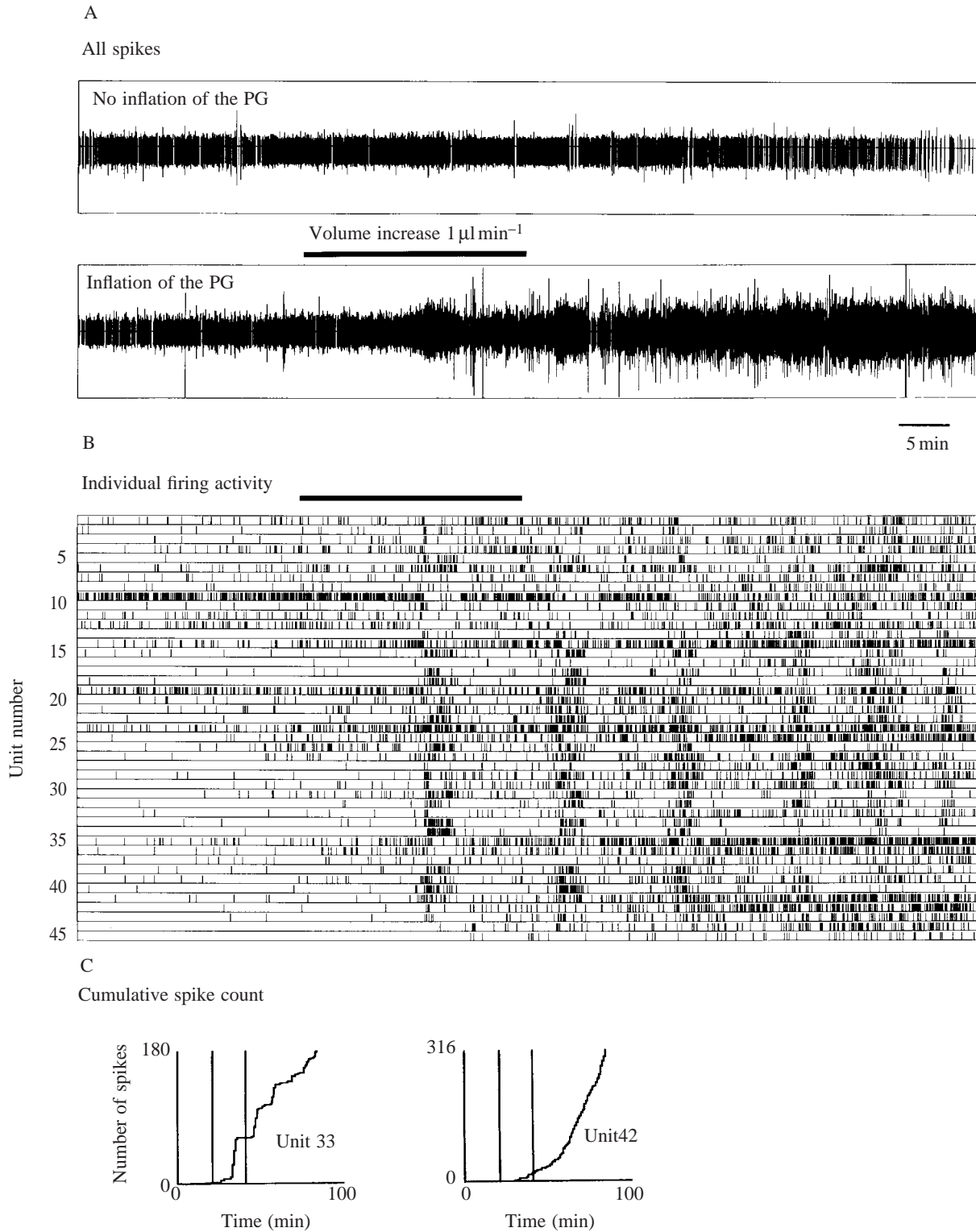


Fig. 7. Electrical recording from the intact nerve NP1 in a semi-intact preparation during a control experiment and during an increase in the volume of the prostate gland. (A) Digitized recording from NP1 without injection of saline into the prostate gland (PG; upper trace) or with injection of saline (bar between traces) in the prostate gland (lower trace). (B) Spike trains of the different units in NP1 recorded during injection of saline into the prostate gland (bar above trace). (C) Cumulative spike counts of two different units in NP1 during the recording. The vertical bars represent the onset and end of saline injection: unit 33 shows step increases in firing activity; unit 42 shows a sustained increase in firing activity.

Table 1. Effect of the injection of saline into the prostate gland on the electrical activity of nerve NP1

Recording	Firing rate (min ⁻¹)		Effect on firing activity	H-value	P
	Before	After			
During volume increase					
NP1	53.9	174.4	Increase	761.51	<0.001
NP1	40.6	110.7	Increase	275.06	<0.001
NP1	28.8	69.8	Increase	284.52	<0.001
NP1	45.7	163.8	Increase	849.94	<0.001
NP1	15.2	66.7	Increase	112.51	<0.001
NP1	29.7	58.4	Increase	5.13	<0.025
NP1	158.85	254.25	Increase	314.2	<0.001
Without volume increase					
NP1	34.5	31.5	None	0.04	NS
NP1	23.1	19.8	Decrease	6.17	<0.025

The sampling period of firing activity before the onset of saline injection is 20 min and after the onset of injection is 50 min. The H-value was calculated using the Kruskal–Wallis test; NS, not significant.

isolation is lower than in control snails. Finally, it was demonstrated that NP1 carries information about the volume of the prostate gland.

The volume of the prostate gland controls male sexual drive

The results of the mass experiments show that a correlation exists between the change in mass of the prostate gland and the change in male sexual drive. The mass of the prostate gland increases during 8 days of social isolation and decreases after copulation as a male. The level of male sexual drive shows an equivalent change under the same conditions: it is high after a period of social isolation and decreases after copulation as a male (Van Duivenboden and Ter Maat, 1985). Since the mass of the prostate gland increases with isolation and decreases with copulation, it is likely that the change in mass is due to a change in the amount of secretory material stored. This change implies a change in volume, and therefore the change in mass is referred to as a change in volume.

The mass of the seminal vesicles does not change during 8 days of social isolation. However, copulation after social isolation does induce a decrease in the mass of the seminal vesicles. This suggests that sperm cells stored in the seminal vesicles are used during copulation. The lack of a significant change in mass during social isolation suggests that, during this period, either no further storage of sperm cells takes place or storage and resorption are in balance. The latter explanation seems to be more appropriate since it has been found that large numbers of the stored sperm cells are phagocytosed in the seminal vesicles (De Jong-Brink, 1984). The mass of the vesicles does not seem to be correlated with the change in male sexual drive.

We found that NP1 forms a nervous connection between the prostate gland and the CNS. The level of male sexual drive in animals with a lesion of this nerve is much lower than in control snails. Although we cannot rule out the possibility that hormonal factors from the prostate gland influence the level of

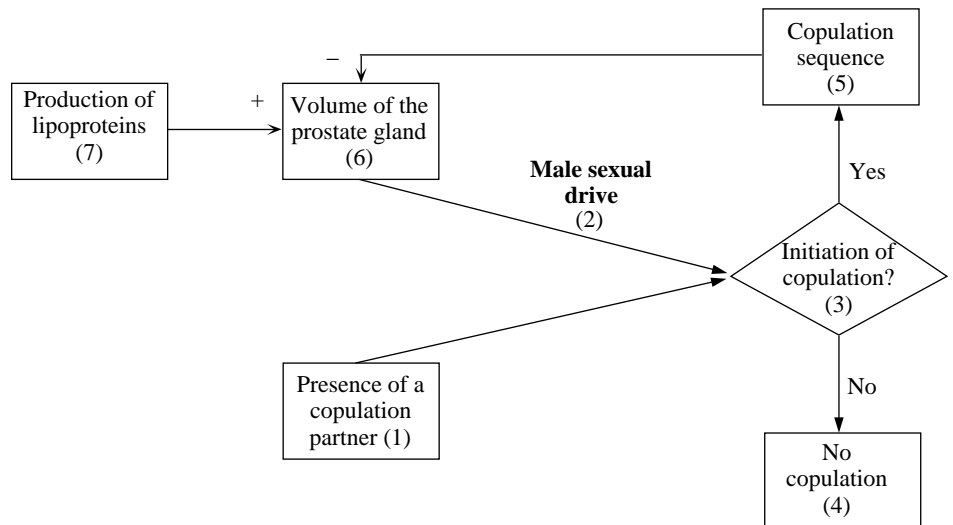


Fig. 8. Model for male sexual drive in *Lymnaea stagnalis*. The initiation of copulation in the male role is dependent on two different variables: male sexual drive and the presence of a copulation partner. The level of male sexual drive is set by the volume of the prostate gland. This volume is regulated by the production and removal of lipoproteins. The numbers indicated in parentheses refer to the description given in the text.

male sexual drive, the results shown here indicate that an intact NP1 is necessary to reach the normal level of male sexual drive after 8 days of isolation.

Extracellular recordings from NP1 show an overall increase in electrical activity during inflation of the prostate gland. Using recordings from the peripheral nerve ends, it was shown that such an increase in activity is generated in the periphery. The increase in firing activity started between 5 and 10 min after the onset of the prostate gland volume increase. After 10 min of saline injection, the prostate gland volume increased by 10 μ l. If the rate of prostate gland volume increase during isolation is linear, this volume increase would be achieved naturally after 4 days of isolation. A period of 4 days of isolation is known to induce an increase in male sexual drive (Van Duivenboden and Ter Maat, 1985). We tested the effect of 8 days of isolation on prostate gland volume and male sexual drive. Together, the data of van Duivenboden and Ter Maat (1985) and the data presented here indicate that an increase of 10 μ l in the volume of the prostate gland can be detected by the snail.

Perception of volume extension

The results described here strongly suggest that stretch-sensing structures are present in the prostate gland. Such structures would be capable of measuring the volume changes in the prostate gland which affect the level of male sexual drive. This mechanism is comparable to the control of satiation by stomach distension (Booth, 1978) and has also been examined in molluscs. In *Aplysia californica*, it has been demonstrated that bulk stimuli induce satiation: in these animals, the mass of the anterior gut contents was found to be correlated with satiation. Injection of gel into the anterior gut caused satiation, and it was suggested that satiation in *Aplysia californica* is mediated by stretch receptors (Susswein and Kupfermann, 1975). In *Lymnaea stagnalis*, neurones have been found in the buccal ganglia that respond to saline injection into the pro-oesophagus. To obtain this response, the pro-oesophagus had to be distended by a factor of 2–3 times its normal size. When a steady distension of this sort was applied for 15–30 s, a burst of action potentials was evoked in the normally silent neurones. This burst lasted for approximately 7 s (Elliott and Benjamin, 1989).

The putative stretch receptors that are responsible for the activity in NP1 after a volume increase of the prostate gland show a sustained response following the volume increase. Future experiments will establish the sensory structures that cause the change in firing activity of NP1 during inflation of the prostate gland.

Model for the male sexual drive

The present findings allow us to formulate a simple model for the control of male sexual drive by the volume of the prostate gland. This model is presented in Fig. 8. The numbers in parentheses in Fig. 8 refer to the features described below. Two demands have to be met for the execution of male copulatory behaviour. First, there has to be a partner present

that can be mounted (1). Second, the male sexual drive has to be sufficiently high to induce copulatory behaviour (2). These two aspects influence whether copulation will occur. If either no copulation partner is present or the male sexual drive is low, there will be no copulation (4). When male sexual drive is high enough and a copulation partner is available, the decision to copulate will be positive and is followed by the execution of the copulatory behaviour sequence (5). The level of male sexual drive is affected by the volume of the prostate gland (6). Two parameters influence this volume. The production of secretory material (7) induces an increase in the volume. Ejaculation during the copulation sequence (5) causes a decrease in the volume. Thus, the combination of production and usage of secretory material in the prostate gland affects the level of male sexual drive in *Lymnaea stagnalis*.

The model implies that male sexual drive can only be observed when there is a potential female partner present that can be mounted. Other studies on motivational aspects of behaviour have used comparable measuring designs. The amount of appetitive male sexual behaviour in the quail *Coturnix japonica* can be measured by the amount of time the male spends in front of a window providing a view of a female (Balthazart *et al.* 1995). Food-arousal can often be measured after animals have received a food stimulus (honeybee *Apis mellifera*: Braun and Bicker, 1992; *Aplysia californica*: Kupfermann *et al.* 1991; *Lymnaea stagnalis*: Tuersley and McCrohan, 1987). Feeding behaviour in several species of the genus *Aplysia* also occurs spontaneously as vacuum activity (reviewed in Kandel, 1976). There have been no reports about spontaneous copulatory behaviour in *Lymnaea stagnalis*. Therefore, it is not known whether male sexual drive can be measured without exposing the animal to a conspecific.

The model for male sexual drive presented here is simple. We do not exclude that other mechanisms can alter the level of male sexual drive in *Lymnaea stagnalis*. In *Aplysia fasciata*, sexual behaviour can be induced by homogenates of the hermaphroditic duct (Susswein and Benny, 1985) and egg masses (Begnoche *et al.* 1996). However, nothing is known about the controlling mechanism that induces the motivational changes. We believe that the present study provides a physiological basis for male sexual drive in molluscs.

We wish to thank Dr M. Kurakawa and P. F. van Soest for critically reading the manuscript. This research was supported by the Foundation for Life Sciences (SLW; grant 805-25-305), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

References

- ADAMO, S. A. AND CHASE, R. (1990). Dissociation of sexual arousal and sexual proclivity in the garden snail *Helix aspersa*. *Behav. neural Biol.* **54**, 115–130.
- BALTHAZART, J., REID, J., ABSIL, P., FOIDART, A. AND BALL, G. F. (1995). Appetitive as well as consummatory aspects of male sexual behaviour in quail are activated by androgens and estrogens. *Behav. Neurosci.* **109**, 485–501.

- BEGNOCHE, V. L., MOORE, S. K., BLUM, N., VAN GILS, C. AND MAYERI, E. (1996). Sign stimulus activates a peptidergic neural system controlling reproductive behavior in *Aplysia*. *J. Neurophysiol.* **75**, 2161–2166.
- BOOTH, D. A. (1978). *Hunger Models: Computable Theory of Feeding Control*. London: Academic Press.
- BRAUN, G. AND BICKER, G. (1992). Habituation of an appetitive reflex in the honeybee. *J. Neurophysiol.* **67**, 588–598.
- DAVIS, W. J., MPITSOS, G. J., PINNEO, M. AND RAM, J. L. (1977). Modification of the behavioral hierarchy of *Pleurobranchaea*. I. Satiation and feeding motivation. *J. comp. Physiol.* **117**, 99–125.
- DE BOER, P. A. C. M., JANSEN, R. F. AND TER MAAT, A. (1996). Copulation in the hermaphroditic snail *Lymnaea stagnalis*: a review. *Invert. Repr. Dev.* **30**, 167–176.
- DE CATANZARO, D. AND GORZALKA, B. B. (1980). Sexual arousal in male mice: effects of brief periods of isolation or grouping. *Behav. neural Biol.* **28**, 442–453.
- DE JONG-BRINK, M. (1984). The regulation of male activity in the gonad of the hermaphrodite basommatophoran snail *Lymnaea stagnalis*. In *Advances of Invertebrate Reproduction*, vol. 3 (ed. W. Engels, W. H. Clark, A. Fischer, P. J. M. Olive and D. F. Went), pp. 213–222. Amsterdam: Elsevier Science Publishers.
- 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.
- ELO, J. E. (1938). Das nervensystem von *Lymnaea stagnalis* (L.). *Lam. Ann. Zool. Vanamo* **6**, 1–40.
- GERAERTS, W. P. M. AND JOOSSE, J. (1984). Freshwater snails (Basommatophora). In *The Mollusca*, vol. 7, *Reproduction* (ed. A. S. Tompa, N. H. Verdonk and J. A. M. van den Biggelaar), pp. 141–207. New York: Academic Press.
- HAMMER, M., BRAUN, G. AND MAUELSHAGEN, J. (1994). Food-induced arousal and nonassociative learning in honeybees: dependence of sensitization on the application site and duration of food stimulation. *Behav. neural Biol.* **62**, 210–223.
- HERMANN, P. M., TER MAAT, A. AND JANSEN, R. F. (1994). The neural control of egg-laying behaviour in the pond snail *Lymnaea stagnalis*: motor control of shell turning. *J. exp. Biol.* **197**, 79–99.
- JANSEN, R. F. AND TER MAAT, A. (1992). Automatic wave form classification of extracellular multineuron recordings. *J. Neurosci. Meth.* **42**, 123–132.
- KANDEL, E. R. (1976). *Cellular Basis of Behaviour*. San Francisco: W. H. Freeman and Company.
- KUPFERMANN, I. (1974). Feeding behavior in *Aplysia*: A simple system for the study of motivation. *Behav. Biol.* **10**, 1–26.
- KUPFERMANN, I., TEYKE, T., ROSEN, S. C. AND WEISS, K. R. (1991). Studies of behavioral state in *Aplysia*. *Biol. Bull. mar. biol. Lab., Woods Hole* **180**, 262–268.
- LI, G. AND CHASE, R. (1995). Correlation of axon projections and peptide immunoreactivity in mesocerebral neurons of the snail *Helix aspersa*. *J. comp. Neurol.* **353**, 9–17.
- MELIS, M. R. AND AGRIOLOS, A. (1995). Dopamine and sexual behavior. *Neurosci. Biobehav. Rev.* **19**, 19–38.
- PETERS, A., STRENG, A. AND MICHIELS, N. K. (1996). Mating behaviour in a hermaphroditic flatworm with reciprocal insemination: do they assess their mates during copulation? *Ethology* **102**, 236–251.
- RUDOLPH, P. H. (1979). The strategy of copulation of *Stagnicola elodes* (Say) (Basommatophora: Lymnaeidae). *Malacologia* **19**, 147–155.
- SOKAL, R. R. AND ROHLF, F. J. (1995). *Biometry*, third edition. New York: W. H. Freeman and Company.
- SUSSWEIN, A. J. AND BENNY, M. (1985). Sexual behavior in *Aplysia fasciata* induced by homogenates of the distal large hermaphroditic duct. *Neurosci. Lett.* **59**, 325–330.
- SUSSWEIN, A. J. AND KUPFERMANN, I. (1975). Localization of bulk stimuli underlying satiation in *Aplysia*. *J. comp. Physiol.* **101**, 309–328.
- SUSSWEIN, A. J., MARKOVICH, S. AND ACHITUV, Y. (1993). Asymmetry of male and female mating in mixed-species groups of *Aplysia fasciata* Poiret and *Aplysia brasiliiana* Rang (Mollusca: Gastropoda). *Israel J. Zool.* **39**, 213–229.
- TINBERGEN, N. (1951). *The Study of Instinct*. London: Oxford University Press.
- TUERSLEY, M. D. (1989). How is food arousal manifested in the pond snail *Lymnaea stagnalis*? An overview. *J. mollusc. Stud.* **55**, 209–216.
- TUERSLEY, M. D. AND MCCROHAN, C. R. (1987). Food arousal in the pond snail, *Lymnaea stagnalis*. *Behav. neural Biol.* **48**, 222–236.
- VAN DER STEEN, W. J., VAN DEN HOVEN, N. P. AND JAGER, J. C. (1969). A method for breeding and studying freshwater snails under continuous water change, with some remarks on growth and reproduction in *Lymnaea stagnalis* (L.). *Neth. J. Zool.* **19**, 131–139.
- VAN DUIVENBODEN, Y. A. AND TER MAAT, A. (1985). Masculinity and receptivity in the hermaphrodite pond snail, *Lymnaea stagnalis*. *Anim. Behav.* **33**, 885–891.
- WILSON, C., NOMIKOS, G. G., COLLU, M. AND FIBIGER, H. C. (1995). Dopaminergic correlates of motivated behaviour: importance of drive. *J. Neurosci.* **15**, 5169–5178.
- ZIV, I., BENNI, M., MARKOVICH, S. AND SUSSWEIN, A. J. (1989). Motivational control of sexual behavior in *Aplysia fasciata*: sequencing and modulation by sexual deprivation and addition of partners. *Behav. neural Biol.* **52**, 180–193.