

## NON-LINEAR DYNAMICS OF NEUROCHEMICAL MODULATION OF MOSQUITO OVIDUCT AND HINDGUT CONTRACTIONS

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### Summary

Microphysiological techniques and computer-based methods of data acquisition and analysis were used to investigate the dynamics of neurochemical action on spontaneous contractions of continuously perfused oviducts and hindguts isolated from female mosquitoes (*Aedes aegypti*). Proctolin, leucomyosuppressin (LMS), serotonin and octopamine, which are known to modulate insect muscle contractions, and a mosquito neuropeptide, *Aedes* Head Peptide I (Aea-HP-I), which inhibits host-seeking behavior, were tested in the bioassays. LMS depressed contractile activity in oviducts at concentrations above  $10^{-12}$  mol l $^{-1}$ , but hindguts did not respond to concentrations below  $10^{-6}$  mol l $^{-1}$ . Hindgut contractions spontaneously restarted in  $10^{-6}$  mol l $^{-1}$  LMS, but only washing out LMS restored activity in oviducts. LMS changed the amplitude of the oviduct contractions, but the dynamics of contraction remained steady. Following recovery of contractions in LMS, hindgut tissues contracted with a more regular pattern.

Serotonin and octopamine had an identical action on oviduct and hindgut tissues. At concentrations greater than

$10^{-8}$  mol l $^{-1}$ , serotonin eliminated the refractory period between contractions and thus increased the contraction frequency of oviducts. Though the contractions appeared chaotic, state-series analysis proved that serotonin-treated oviducts contracted rhythmically. Above  $10^{-6}$  mol l $^{-1}$ , serotonin extinguished normal hindgut peristalsis and induced regular fluttering of the anteriormost portion of the hindgut, an effect possibly related to diuresis following the blood meal.

Proctolin failed to stimulate both oviduct and hindgut contractions at concentrations up to  $10^{-6}$  mol l $^{-1}$ , but at  $10^{-8}$  mol l $^{-1}$  induced contractions of cricket hindgut preparations. Aea-HP-I had no effect on either tissue at  $10^{-6}$  mol l $^{-1}$  or lower concentrations.

State-series analysis, based on simple manipulations of experimental data, permitted direct observation of the dynamics of oviduct and hindgut contractile activity.

Key words: *Aedes aegypti*, mosquito, peptides, contractile bioassay, serotonin, octopamine, proctolin, leucomyosuppressin, *Aedes* Head Peptide I, chaos theory.

### Introduction

As the identification and characterization of neurochemical messengers in mosquitoes proceeds, critical insight into the specific effects and modes of action of these molecules is best provided by homologous bioassays (Matsumoto *et al.* 1989; Brown and Lea, 1990; Novak and Rowley, 1994; Brown *et al.* 1994; Hayes *et al.* 1994; Veenstra, 1994). Visceral and skeletal muscle systems are used commonly to characterize the properties of diverse neurochemicals on orthopteran insects (Schoofs *et al.* 1993; Puiroux *et al.* 1993; Evans, 1994). Additionally, orthopteran systems have been used as heterologous bioassays to isolate neuropeptides from other insect species (Holman *et al.* 1991). Similar bioassays have been described for three dipteran species, the horsefly (*Tabanus sulcifrons*, Cook and Meola, 1978), the onion fly (*Delia antiqua*, Mowry *et al.* 1987) and the stable fly (*Stomoxys calcitrans*; Cook *et al.* 1990), and used for the evaluation of neurochemical effects (Cook and Meola, 1978; Cook and Wagner, 1992).

The goal of our work was to develop a bioassay system appropriate for measuring the myogenic contractions of the oviducts (approximately 700  $\mu$ m long) and hindguts (approximately 2.9 mm long) of the yellow fever mosquito, *Aedes aegypti*, and to validate this system with known compounds. With this bioassay, we plan to monitor the isolation of neuropeptides that affect digestive and reproductive processes in *A. aegypti* and to investigate the dynamics of any responses to endogenous factors.

The experimental techniques and instrumentation were modified from those of Mowry *et al.* (1987) and designed to acquire data digitally for enhanced statistical analyses of the minute movements and forces characteristic of mosquito muscles. Proctolin, leucomyosuppressin, serotonin and octopamine, which are known to modulate insect muscle contractions, were used to validate the techniques and instrumentation. In addition, a mosquito neuropeptide, *Aedes* Head Peptide I, which inhibits host-seeking behavior of

females (Brown *et al.* 1994), was tested for specific effects on these muscle systems.

Visualised as a function of time, many insect muscle contractions display no apparent pattern *in vitro*. We considered that these isolated tissue preparations might approximate a nonlinear physical system, and chose to apply techniques used for analysis of nonlinear dynamics. Based on elements of chaos theory (Ditto and Pecora, 1993; Elbert *et al.* 1994), our analyses of the effects of the neurochemicals provided insights into the basic biological properties of the oviduct and hindgut contractile systems in *A. aegypti* and allowed comparisons between insect species.

## Materials and methods

### Buffers and experimental solutions

Preparations were perfused in a freshly prepared buffered saline containing, in  $\text{mmol l}^{-1}$ :  $\text{CaCl}_2$ , 1.8;  $\text{KCl}$ , 3.4;  $\text{NaCl}$ , 150; Hepes, 25; glucose, 5;  $\text{MgCl}_2$ , 0.6; and  $\text{NaHCO}_3$ , 1.8. This buffer was brought to pH 7.2 with  $\text{NaOH}$ . Solutions used in experiments were continuously bubbled with air. Experiments were conducted at ambient laboratory temperatures ( $22\text{--}25^\circ\text{C}$ ). Test chemicals were completely solubilised in dimethylformamide (DMF), and the resulting mixture was diluted with buffered saline to 20% DMF for serial dilutions and frozen at  $-80^\circ\text{C}$  for up to 4 months. Tissues, treated with a solution of 1% DMF in the buffer above, did not differ in any apparent way from tissues perfused with buffer alone; usually perfusates contained less than 0.02% DMF. Leucomyosuppressin (LMS) was purchased from Peninsula Laboratories (Belmont, CA, USA), and proctolin, serotonin (5-hydroxytryptamine) and octopamine from Sigma. *Aedes* Head Peptide I (Aea-HP-I) was synthesized at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA, USA).

### Experimental preparations

*Aedes aegypti* females, 3–5 days post-eclosion, were maintained on a 10% sugar solution. Mosquitoes were chilled over ice prior to dissection of the tissues. The abdomen of the mosquito was gently squeezed with forceps to distend slightly the posteriormost segments. The last apparent abdominal segment, including the terminalia and terminal abdominal ganglion, was cut off. The intersegmental membrane of the penultimate segment was torn slightly with fine forceps. The edges of this tear were grasped with two pairs of forceps, and the integument pulled apart to expose the gut. The anterior portion of the abdomen was again squeezed gently with forceps to force the gut and ovaries towards the posterior. The required tissue was removed, stripped of fat body and tracheae, and transferred to the perfusion well of the experimental apparatus described below.

A similar technique was used for tissue preparations of the domestic cricket *Acheta domestica* L., which were purchased from Monroe Cricket and Grub Farm (Monroe GA, USA).

### Bioassay apparatus

We modified the apparatus described by Mowry *et al.* (1987) for their studies of oviduct contractions of the onion fly *Delia antiqua* (Meigen).

The incubation chamber, which was molded in a 5 cm plastic Petri dish from Sylgard 184 silicone elastomer, is shown in Fig. 1. A Teflon form was used to create the  $50\text{ }\mu\text{l}$  perfusion well. Buffer supply and draining tubes were embedded in the resin prior to hardening. After the elastomer had solidified, the incubation chamber was removed from the Petri dish.

Suction pipettes were prepared by softening polyethylene tubing (0.965 mm or 2.08 mm outside diameter) over a flame and drawing it out to an outside diameter of  $150\text{--}200\text{ }\mu\text{m}$ . A fixed suction pipette was passed through the elastomer into the perfusion well with the aid of a hypodermic needle. For the mobile pipette, drawn polyethylene tubing was passed through a 1.5 inch  $\times$  25 gauge hypodermic needle, which was then bent at a right angle about 8 mm from the tip. A 1 mm  $\times$  4 mm flag cut from 0.001 inch (25  $\mu\text{m}$ ) aluminum shim stock (Shim in a Can, Shop-Aid, Inc.) was cemented with cyanoacrylate adhesive about 8 mm from the free end of the mobile pipette. The needle was attached to a tuberculin syringe, which was then mounted in a micromanipulator.

For hindgut preparations, the rectum was sucked into the fixed pipette, where it was held by vacuum, and the mobile pipette was attached to the tissue just anterior to the pyloric valve. For oviduct preparations, the spermathecae were aspirated into the fixed pipette, where they lodged near the orifice. The mobile pipette was attached to the ovary just distal to the oviduct, so that contractions of a single oviduct were recorded. In both cases, tissues were allowed to equilibrate for 30–60 min prior to the application of test compounds.

We estimated the amount of force required to move the mobile pipette by positioning a pipette in a micromanipulator

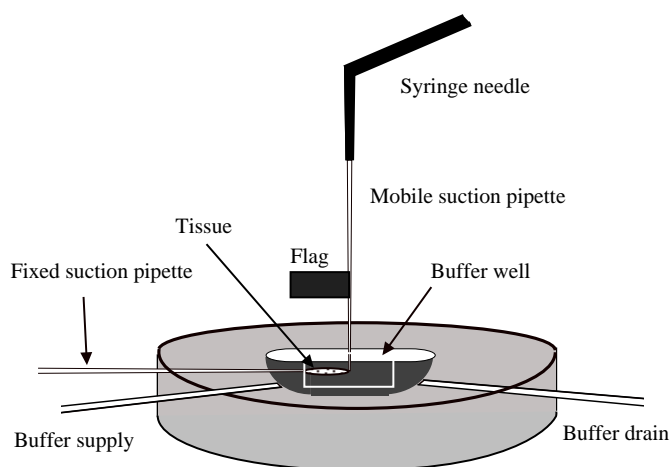


Fig. 1. Perfusion chamber used for hindgut and oviduct contraction assays. The tissue is held by suction between fixed and mobile polyethylene pipettes. Tissue contractions pull the aluminum foil flag in and out of the path of a break-beam photoemitter/photodetector pair (not shown), and the resulting voltage is recorded. The preparation is continuously perfused with a peristaltic pump.

and letting the pipette rest on the pan of a microbalance sensitive to  $1\text{ }\mu\text{g}$ . The pipette was moved  $100\text{ }\mu\text{m}$ , so that it pushed on the pan in the same way that it would be bent by a contracting tissue, and the mass was recorded. We estimated that the mass required to displace the mobile pipette was in the range  $30\text{--}100\text{ }\mu\text{g}$ , which is about  $1.2\text{--}4\%$  of the approximately  $2.5\text{ mg}$  wet mass of a live female *A. aegypti*.

Displacement of the flag attached to the mobile pipette was measured with a break-beam photoemitter/photodetector apparatus similar to that described previously (Mowry *et al.* 1987). The detector was mounted on a rod attached to a micromanipulator. The signal was acquired digitally with a computer-based data-acquisition system consisting of a National Instruments NB-MIO-16X data-acquisition board installed in an Apple Macintosh Quadra 800 computer. Software for data acquisition was written in the Labview programming language (National Instruments, Austin, TX, USA). The data-acquisition system also controlled a solenoid valve which switched treatment solutions. Tissues were continuously perfused at a constant rate of about  $100\text{ }\mu\text{l min}^{-1}$  with a peristaltic pump (Minipuls-3, Gilson Instrument).

A bioassay, similar to that described for mosquitoes, was also used with crickets, *Acheta domestica*. For these assays, the well of the incubation chamber contained  $0.9\text{ ml}$  and was perfused at a rate of  $0.42\text{ ml min}^{-1}$ . The suction pipettes were constructed with polyethylene tubing of  $0.5\text{ mm}$  inside diameter.

#### Data acquisition and processing

Statistical analysis of records was performed with DataDesk; curves were drawn through discrete data using a treewss smoother (Velleman, 1992).

Recordings of contracting oviducts were made at  $20$  or  $50\text{ Hz}$ ; for hindguts, the sampling rate was set at  $10$  or  $20\text{ Hz}$ . The data were acquired digitally, as a two-dimensional array of time and voltage values, corresponding to the transducer output. Displacement was measured in units of volts; the relationship between voltage and displacement was linear. We did not determine whether the transducer response was linear with respect to force.

Using Labview, we wrote programs that computed intercontraction intervals, contraction amplitudes and the velocity of a contracting muscle at any point in its contraction cycle. Intercontraction intervals were computed by constructing an array of times at which each contraction peak occurred. The previous (absolute) time value was subtracted from the current value; in other words, the array element  $I_{n-1}$  was subtracted from the element  $I_n$  to give the intercontraction interval. Computationally this was achieved by duplicating the array of time values, shifting the duplicated array down one place and subtracting it from the original array.

The amplitude of each contraction was determined by subtracting the minimum displacement of a contraction from the maximal displacement. The velocity of the contracting muscle was determined in a manner similar to that for intercontraction intervals. The previous voltage value,  $V_{n-1}$ ,

was subtracted from the current voltage value,  $V_n$ . The resulting value was then divided by the sample interval to give the rate of change in voltage units per second. From the velocity information, we estimated the proportion of each complete contraction-relaxation cycle during which the tissue was contracting. This was done by determining the proportion of time in a contraction cycle where the velocity was greater than zero, which occurs only when the tissue is contracting.

State-series graphs were constructed by plotting the velocity of the contracting muscle at a discrete position in the contraction cycle against the same discrete position. The voltage itself corresponds to the absolute position of the muscle in the contraction cycle.

## Results

Most of the hindgut and oviduct preparations began to contract spontaneously after they were isolated from a mosquito and mounted in the perfusion well. This indicates that these contractile tissues can generate their own myogenic rhythms. These rhythms, however, can be controlled by factors extrinsic to the tissue, such as the neurochemicals used in our experiments. Irregular peristaltic contractions, in which the entire tissue contracted, typified hindgut activity. Regular, sometimes synchronous, contractions were typical of isolated oviducts.

#### *Leucomyosuppressin modulation of oviduct contractions*

Leucomyosuppressin decreased the amplitude of oviduct contractions, but did not have any apparent effects on the intercontraction intervals. Of the  $10$  preparations tested, three responded at LMS concentrations of  $10^{-12}\text{ mol l}^{-1}$ , two responded at  $10^{-10}\text{ mol l}^{-1}$  and a single preparation responded at  $10^{-8}\text{ mol l}^{-1}$ . These preparations responded to LMS up to the maximum concentration used,  $10^{-6}\text{ mol l}^{-1}$ . The remaining four preparations did not respond to LMS in any apparent manner. Washing the preparation with buffer reversed the suppressive effects of LMS; oviducts never spontaneously recovered control levels of activity in the presence of LMS.

The results and analysis of a typical experiment with LMS are shown in Figs 2 and 3. Fig. 2A shows oviduct contractions during the first  $600\text{ s}$  of an experiment in which an oviduct preparation was treated with  $10^{-8}\text{ mol l}^{-1}$  LMS. Fig. 2B reports the intercontraction intervals; the amplitude of individual contractions is shown in Fig. 2C.

From these traces, it is apparent that leucomyosuppressin decreases the amplitude of the oviduct contractions. At the time of maximum effect ( $330\text{ s}$ ) prior to the buffer wash, the amplitude ( $0.07\pm 0.036\text{ V}$ , mean  $\pm$  S.E.M.,  $N=53$  contractions) is depressed compared with the contraction amplitude of the preparation under the buffer treatment alone ( $0.502\pm 0.126\text{ V}$ ,  $N=34$ ) ( $P<0.0001$ , pooled  $t$ -test,  $85\text{ d.f.}$ ). The intercontraction intervals could not be measured during the period of maximum LMS effect because the contractions could not be reliably distinguished from the baseline, but from  $200$  to  $215\text{ s}$  the mean intercontraction interval ( $1.60\pm 0.4\text{ s}$ ,  $N=10$ ) did not differ from

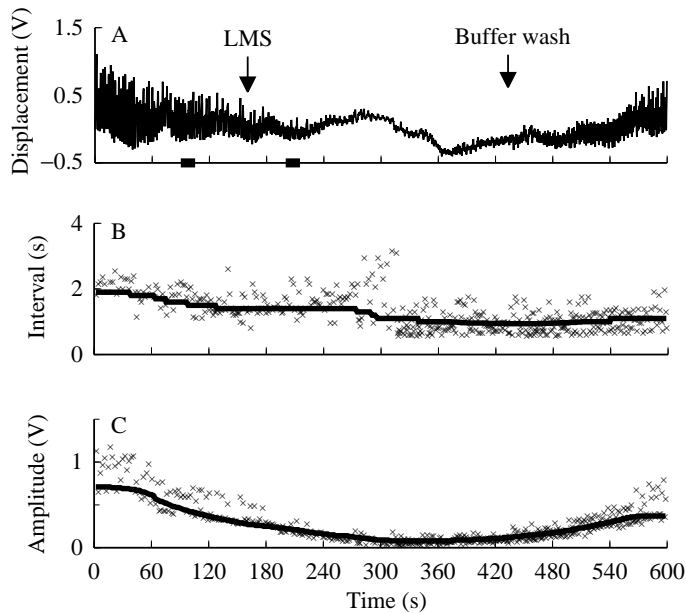


Fig. 2. Effects of  $10^{-8} \text{ mol l}^{-1}$  leucomyosuppressin (LMS) on oviduct contractions of *Aedes aegypti*. (A) Trace of oviduct displacement. Perfusion with  $10^{-8} \text{ mol l}^{-1}$  LMS. Heavy bars on the time axis indicate regions expanded in Fig. 3. (B) Intercontraction intervals. Midway through the recording the contractions are suppressed; they cannot be differentiated from the baseline. This causes the apparent increase in the intervals between 240 and 320 s. (C) Amplitude of the individual oviduct contractions in response to the LMS treatment.

the mean intercontraction interval ( $1.88 \pm 0.32 \text{ s}$ ,  $N=9$ ) when the tissue was perfused with buffer alone (90–105 s).

A detailed analysis of the dynamics of the response to LMS by a single oviduct is shown in Fig. 3, which shows enlarged sections from the regions of the record indicated by bold lines on the time axis of Fig. 2A. The top row of traces (Fig. 3A,B) shows time-series plots; these are analogous to the traces obtained with a conventional chart recorder. The traces show that  $10^{-8} \text{ mol l}^{-1}$  LMS reduces the amplitude of the contractions.

The middle row of figures, Fig. 3C,D, shows the relative velocity of the contracting oviduct at a specific time. In these plots, when velocity is greater than zero, the tissue is contracting, when velocity is zero the tissue is static, and when velocity is less than zero the oviduct is relaxing. In buffer (Fig. 3C), the relaxation phase closely follows the contraction phase, resulting in what is essentially a sawtooth wave. Under the influence of  $10^{-8} \text{ mol l}^{-1}$  LMS, this contraction pattern is repeated, though it is clear that the maximum velocity of the individual contractions is reduced.

Fig. 3E,F presents state-series plots of the data, where the velocity of the contracting oviduct at a discrete position is plotted against that position in the contraction cycle. Under control conditions, the contracting oviduct produces the state-series plot shown in Fig. 3E. The tissue produces a similar pattern in response to the LMS treatment, though the amplitude of the contractions is diminished (Fig. 3F).

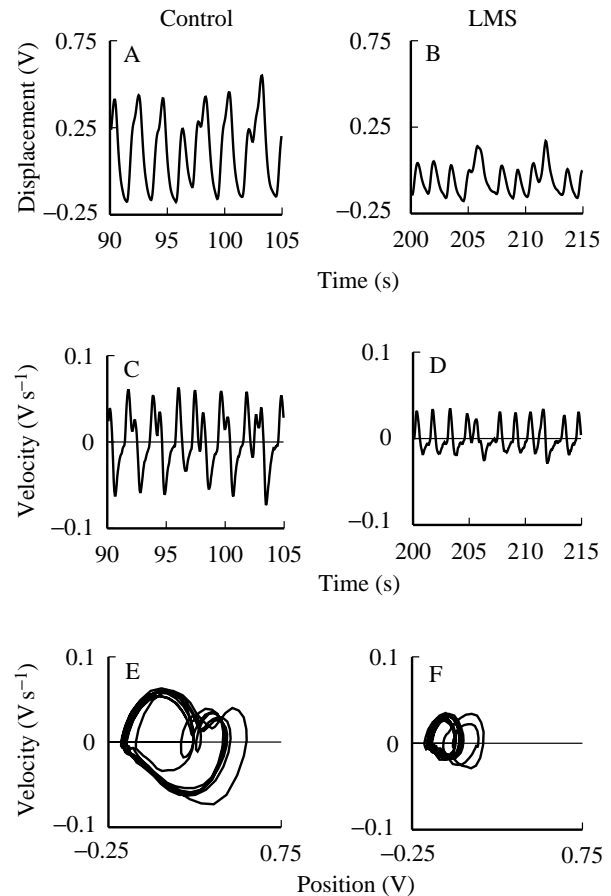


Fig. 3. Detailed analysis of regions indicated in Fig. 2. (A,B) Oviduct displacement in response to  $10^{-8} \text{ mol l}^{-1}$  LMS. (C,D) Profile of contraction velocity, reported as  $\text{V s}^{-1}$ , against time. (E,F) State-series plots. The velocity of the contracting tissue at a discrete position is plotted against that position.

As illustrated in Fig. 4, which shows traces of three separate experiments, the proportion of time an oviduct is contracting (i.e. velocity  $> 0$ ) does not change in response to LMS.

#### Effects of leucomyosuppressin on the hindgut

Leucomyosuppressin suppressed hindgut contractions at a concentration of  $10^{-6} \text{ mol l}^{-1}$ ; however, the response was transient and contractions returned to near control levels spontaneously. Six hindgut preparations were studied; four of these responded to LMS at  $10^{-6} \text{ mol l}^{-1}$ , but not lower concentrations, and two preparations did not respond to LMS at a maximum concentration of  $10^{-8} \text{ mol l}^{-1}$  in one case or  $10^{-6} \text{ mol l}^{-1}$  in the other.

The results of one experiment, in which a hindgut preparation was perfused with  $10^{-6} \text{ mol l}^{-1}$  LMS, are shown in Fig. 5. Leucomyosuppressin clearly reduced the amplitude of the hindgut contractions, as shown in Fig. 5C, but this change is temporary. About 1 min after exposure to LMS, the hindgut contractions cease. However, in contrast to the effect of LMS on oviducts, the hindgut contractions recover

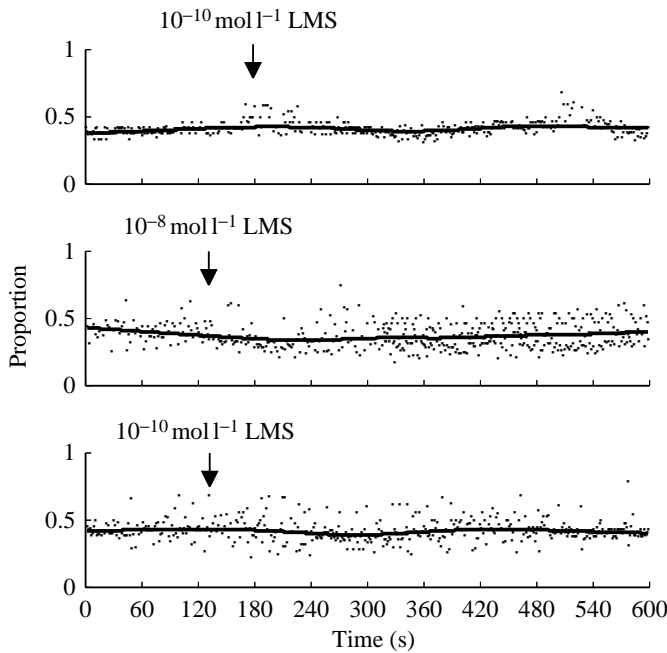


Fig. 4. Proportion of each contraction-relaxation cycle devoted to contraction. Three separate experiments are shown.

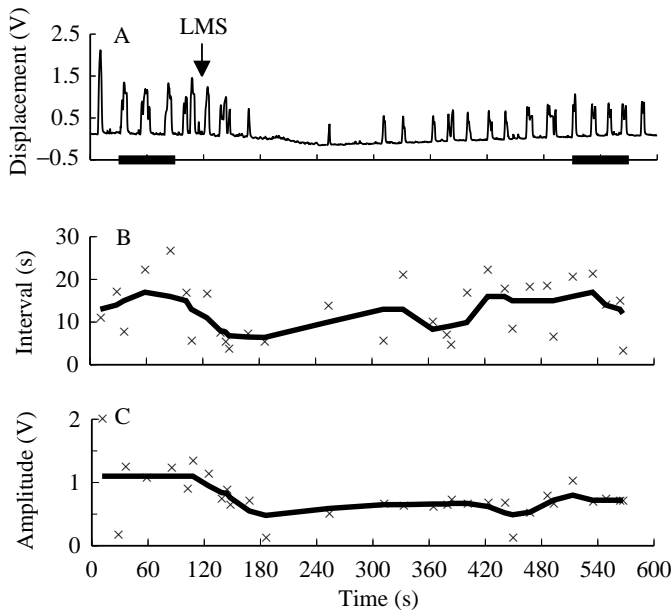


Fig. 5. Response of hindgut tissues to  $10^{-6} \text{ mol l}^{-1}$  LMS. (A) Perfusion of hindgut tissues with  $10^{-6} \text{ mol l}^{-1}$  LMS. Bars indicate regions shown in detail in Fig. 6. (B) Intercontraction intervals. There is not a statistically significant difference in the intercontraction intervals between the 0–120 s and 480–600 s ranges. (C) Amplitude of individual hindgut contractions in response to LMS.

spontaneously 3–4 min after exposure even in the presence of  $10^{-6} \text{ mol l}^{-1}$  LMS.

Because LMS temporarily shuts off the hindgut contractions, it changes the shape of the plot of contraction intervals. In both control and experimental conditions, hindgut

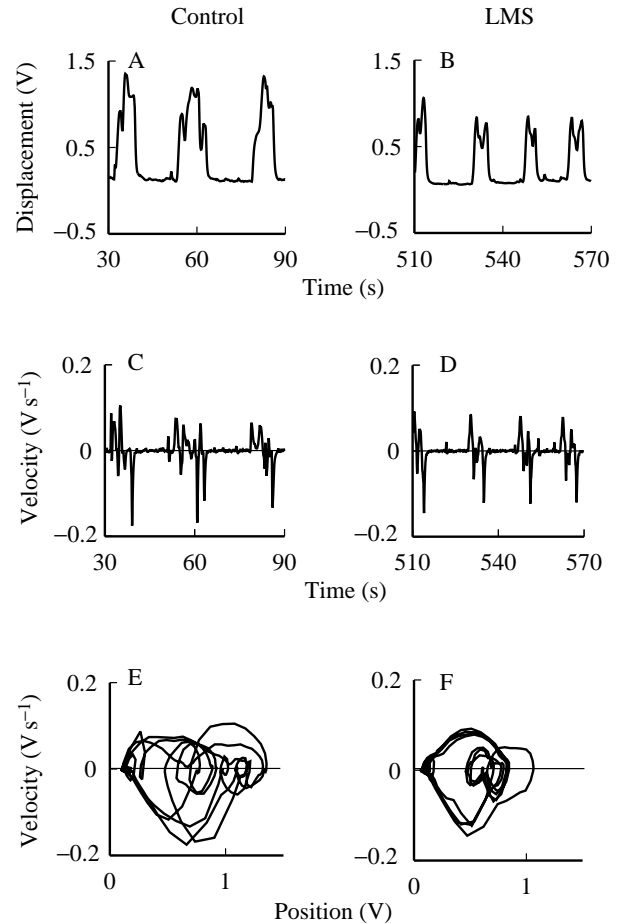


Fig. 6. Analysis of ranges indicated in Fig. 5. (A,B) Displacement of tissue in control conditions and after contractions have restarted in the presence of  $10^{-6} \text{ mol l}^{-1}$  LMS. (C,D) Velocity profiles of hindgut contractions. (E,F) State-series analysis of hindgut contractions.

tissues contract at irregular intervals, without any apparent pattern (Fig. 6A–D). A pooled *t*-test comparing means of intercontraction intervals during the first minute and last two minutes of the experiment showed that there is no difference between these time spans. But the state-series plots, shown in Fig. 6E,F, indicate that LMS causes the dynamics of the contraction cycle to become more regular, even though this feature is not evident on the chart recorder traces.

#### *Effects of serotonin and octopamine on the oviduct*

Each of the four oviduct preparations studied responded to treatment with serotonin at  $10^{-8} \text{ mol l}^{-1}$ , and at  $10^{-6} \text{ mol l}^{-1}$  with changes in tonus, a decrease in the intercontraction interval and changes in the amplitude of contractions. None of these changes was apparent at  $10^{-10} \text{ mol l}^{-1}$  or lower concentrations. Washing the preparations with buffer completely reversed the effects of serotonin on oviduct contractions (data not shown). Octopamine produced a response identical to that of serotonin, at the same concentrations (data not shown).

The results and analysis of a typical experiment are

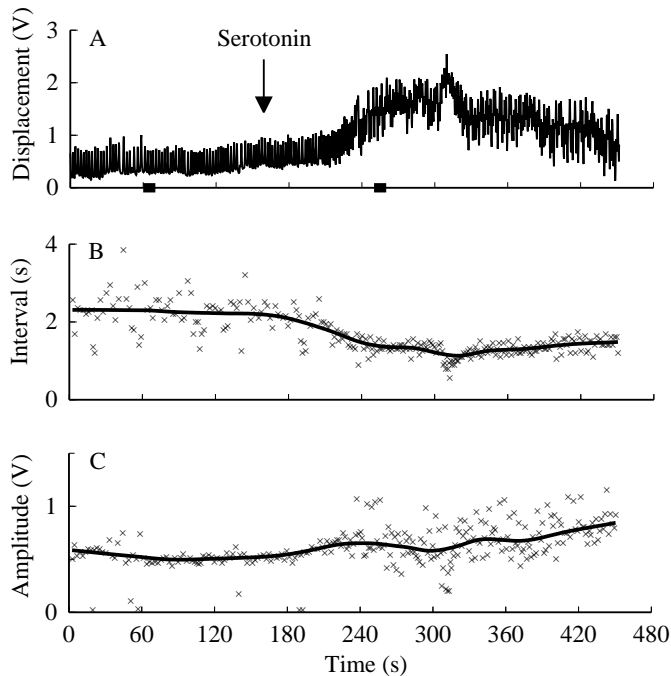


Fig. 7. Response of an *Aedes aegypti* oviduct to  $10^{-8} \text{ mol l}^{-1}$  serotonin. (A) Serotonin increases the basal tension of the oviduct and causes the tissue to contract in an irregular manner. Bars indicate areas subjected to detailed analysis in Fig. 8. (B) Intercontraction intervals. (C) Amplitudes of individual contractions.

presented in Fig. 7. The trace of displacement (Fig. 7A) shows that approximately 90 s after the addition of the serotonin, the tonus of the oviduct increased. The interval between contractions of this oviduct decreased (Fig. 7B) from  $2.2 \pm 0.56$  s for the first 60 s of the experiment to  $1.4 \pm 0.16$  s for the interval from 360 to 420 s ( $P < 0.0001$ , 26 d.f.). Serotonin also increased the contraction amplitude, as shown in Fig. 7C, from  $0.57 \pm 0.07$  V in the buffer to  $0.72 \pm 0.16$  V after treatment with the neurotransmitter ( $P < 0.0001$ , 65 d.f.).

A detailed analysis of these experimental recordings is presented in Fig. 8. These panels show enlarged portions, indicated by the bars on Fig. 7A, of the recordings of control and serotonin treatments. In buffer alone (Fig. 8A), the oviduct contracts with a simple, rhythmic pattern, unlike the complex pattern (Fig. 8B) induced by serotonin. Plots of the velocity of oviduct contractions (Fig. 8C,D) reveal that the serotonin treatment eliminates the resting phase of the contraction cycle, visible at the interval in Fig. 8C when the contraction velocity is zero. The effect of serotonin treatment is thus to increase the rate at which the oviduct contracts. In addition, the serotonin treatment increases the maximum velocity achieved during each contraction cycle (from  $1.29 \pm 0.14 \text{ V s}^{-1}$  to  $1.60 \pm 0.3 \text{ V s}^{-1}$ ,  $P < 0.0001$ , pooled *t*-test, 66 d.f.). It is interesting to note that even though the position of the oviduct varies throughout the contraction cycle (the shifting maxima in Fig. 8B), the profile of velocity against time (Fig. 8D) during a contraction (velocity  $> 0$ ) remains virtually constant.

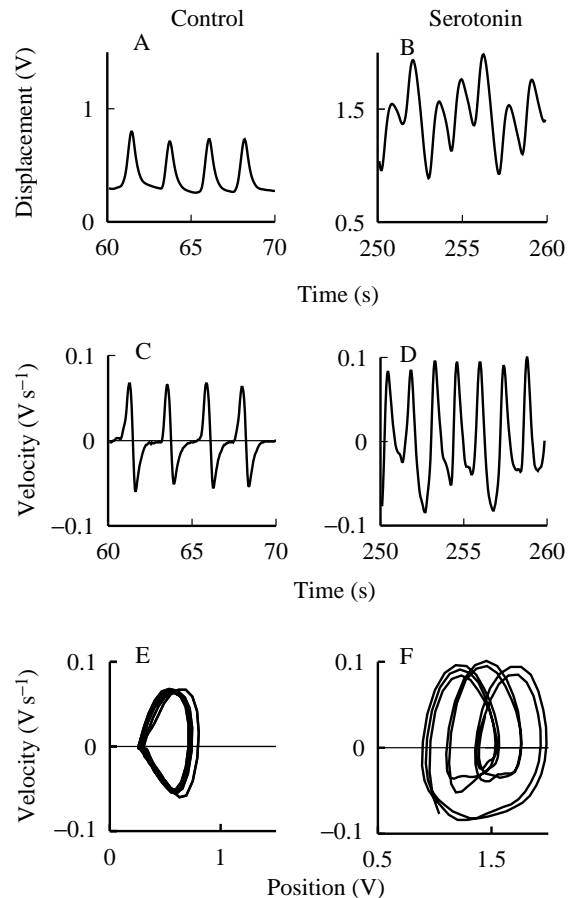


Fig. 8. Detailed analysis of regions indicated in Fig. 7A. (A,B) Displacement. (C,D) Velocity profiles. In buffer, each contraction-relaxation cycle is followed by a resting phase, during which velocity is zero. Serotonin treatment slightly increases the velocity at which the tissue contracts and removes the resting phase. (E,F) State-series analysis of oviduct contractions in control buffer and in response to  $10^{-8} \text{ mol l}^{-1}$  serotonin.

State-series plots of the oviduct contractions in buffer show that the contraction cycle is regular (Fig. 8E). Reflecting both the increased amplitude and velocity of the contractions of the oviduct in response to serotonin, the state-series plot in Fig. 8F covers a greater area than the one in Fig. 8E. That this trace is more complex is due to the shifts in the starting position of each contraction cycle, so that the contractions are operating in different regions of state-space. However, the shapes of the traces during the contraction phase of each cycle, where velocity  $> 0$ , in Fig. 8F are similar.

With the dissecting microscope, we observed the response of the oviduct to the serotonin treatment. In buffer, the oviduct appeared to contract regularly and rhythmically. Treatment with  $10^{-8} \text{ mol l}^{-1}$  serotonin caused the tissue to contract in a rapid and seemingly uncoordinated manner, with a new contraction cycle beginning before the oviduct had completely relaxed.

*Effects of serotonin and octopamine on the hindgut*  
Contraction patterns of the five hindgut preparations tested

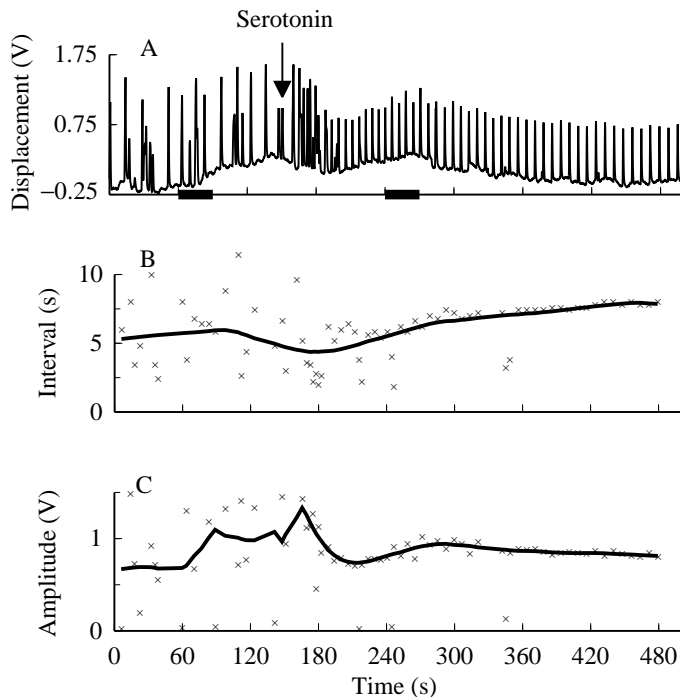


Fig. 9. Response of *Aedes aegypti* hindgut to  $10^{-6} \text{ mol l}^{-1}$  serotonin. (A) Displacement of hindgut contractions. Bars indicate regions subjected to detailed analysis in Fig. 10. (B) Intercontraction intervals. (C) Amplitude of hindgut contractions.

were completely altered by treatment with serotonin. Each of the five hindgut preparations tested responded to  $10^{-6} \text{ mol l}^{-1}$  serotonin; at lower concentrations, this neurochemical did not cause any major changes in the contraction patterns. The effects of the serotonin treatment were completely reversible on washing with buffer. Each of four hindgut preparations tested with octopamine responded in a manner identical to its response to serotonin.

The effects of  $10^{-6} \text{ mol l}^{-1}$  serotonin on an individual hindgut preparation are illustrated in Fig. 9A. Shortly after treatment with  $10^{-6} \text{ mol l}^{-1}$  serotonin, the irregular contractions of the hindgut were replaced by regular contractions of smaller amplitude. In buffer, the tissue contractions show a broad distribution of intervals (Fig. 9B); the mean time between these intervals was  $5.8 \pm 2.1 \text{ s}$  ( $N=19$ ). In  $10^{-6} \text{ mol l}^{-1}$  serotonin, the mean interval between contractions became an almost constant  $7.3 \pm 0.63 \text{ s}$  ( $N=30$ ). Serotonin also stabilized the amplitude of the contractions. Fig. 9C shows that the mean amplitude of the contractions in the serotonin treatment is  $0.83 \pm 0.14 \text{ V}$ , compared with  $0.83 \pm 0.53 \text{ V}$  in the buffer.

The changes in the hindgut contraction patterns after treatment with serotonin are further illustrated in Fig. 10, which presents enlargements of the sections of the recordings shown in Fig. 9. Treatment with serotonin changes the irregular hindgut contraction pattern seen in Fig. 10A to one characterised by an apparently rhythmic set of contractions of similar amplitude, as shown in Fig. 10B. Under control

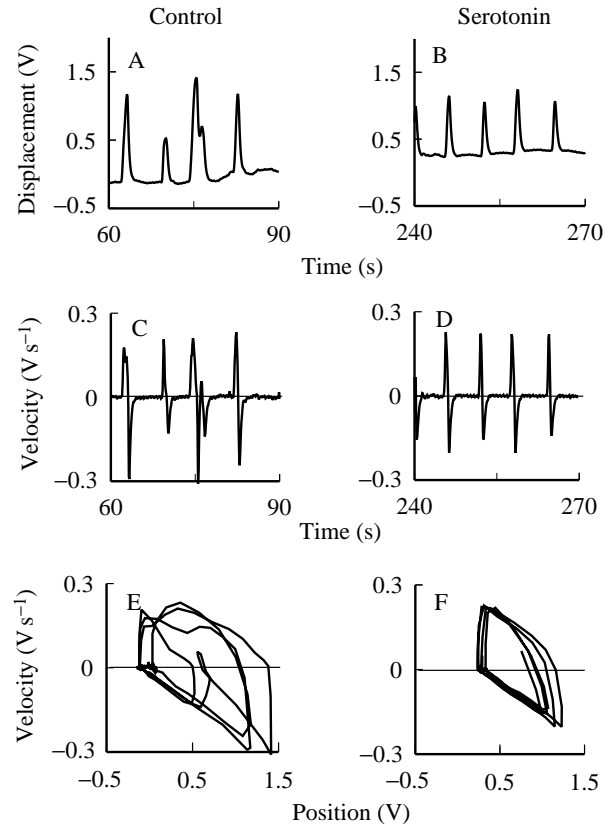


Fig. 10. Detailed analysis of regions indicated in Fig. 9A. (A,B) Displacement of hindgut contractions. (C,D) Velocity profiles. (E,F) State-series analysis of hindgut contractions in control buffer and in response to  $10^{-6} \text{ mol l}^{-1}$  serotonin.

conditions, the velocity profile (Fig. 10C) is not rhythmic, but treatment with serotonin produces the regular velocity profile shown in Fig. 10D. In this trace, the apparently regular contractions are followed immediately by relaxation of the hindgut and a pause. State-series analysis of these traces, shown for the buffer control in Fig. 10E and for a serotonin-treated hindgut in Fig. 10F, demonstrates that serotonin causes the hindgut contractions to shift to a regular contraction pattern from the irregular pattern shown under control conditions.

Microscopic observations revealed that serotonin completely changed the dynamics of the hindgut contractions. In buffer, waves of peristaltic contraction pass down the entire hindgut at irregular intervals. Serotonin suppresses these peristaltic waves and stimulates rhythmic contractions in the region of the pyloric sphincter, just posterior to the insertion of the Malpighian tubules; there is no contraction in the posterior region of the hindgut.

Parallel experiments with octopamine produced an identical response to that of serotonin, at the same concentration, in three oviduct preparations.

#### *Effects of proctolin on mosquito and cricket tissues*

Proctolin, tested on *A. aegypti* tissues at concentrations from  $10^{-10}$  to  $10^{-6} \text{ mol l}^{-1}$  on nine oviduct preparations and

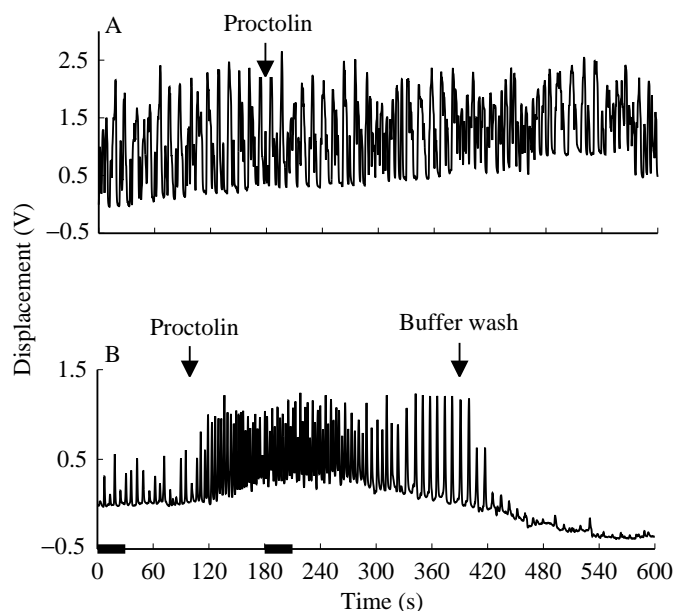


Fig. 11. Response of insect hindgut tissues to  $10^{-8} \text{ mol l}^{-1}$  proctolin. (A) Hindgut of *Aedes aegypti*. (B) Hindgut contractions of the cricket *Acheta domestica*. Bars indicate regions expanded in Fig. 13.

ten hindgut preparations, did not produce any detectable response. Experiments with cricket tissues were conducted with proctolin from the same batch as that used for the mosquito experiments. Of three preparations made with cricket hindguts, one responded to proctolin at  $10^{-9} \text{ mol l}^{-1}$  and two responded at  $10^{-8} \text{ mol l}^{-1}$ . The response was reversible with washing.

Fig. 11 compares the response to proctolin of an *A. aegypti* hindgut (Fig. 11A) and an *Acheta domestica* hindgut (Fig. 11B). In the proctolin treatment, both the amplitude and frequency of hindgut contractions of the cricket hindgut increase, while there are no effects visible on the mosquito hindgut. This species-specific response is apparent in Fig. 12. In the presence of proctolin, cricket hindguts clearly show a decrease in intercontraction intervals (Fig. 12A) and an increase in the amplitude of the contractions (Fig. 12C). Mosquito hindguts do not show these patterns (Fig. 12B,D). The proportion of each contraction cycle during which the cricket hindgut contracts at first increases in response to proctolin, but later decreases to initial levels (Fig. 12E). The mosquito hindgut shows no response (Fig. 12F).

Analysis of the recordings of the *Acheta domestica* hindgut response shows that proctolin increases both the frequency and amplitude of contractions (Fig. 13). Velocity profiles of the traces indicate that contraction-relaxation cycles are followed by a resting phase in the control, while the proctolin treatment increases the velocity of the contraction and removes the quiescent phase (Fig. 13C,D). State-series plots (Fig. 13E,F) show that the contraction cycle in the control buffer is irregular, while proctolin increases the number of contractions and preserves their periodicity.

#### Effects of Aea-HP-I on mosquito tissues

At concentrations from  $10^{-15}$  to  $10^{-6} \text{ mol l}^{-1}$ , Aea-HP-I had no effects on the four oviduct preparations and six hindgut preparations tested.

### Discussion

We have developed bioassays, combining microforce transducers and data-acquisition techniques, to detect effects of neurochemicals on the spontaneous contractions of oviducts and hindguts isolated from female mosquitoes. In our experimental system, less than 5 % of the 2.5 mg mass of the adult female mosquito produces enough force for a full-scale response. Other contraction bioassays, such as those for *Stomoxys calcitrans* (Cook and Wagner, 1992), may put tension to an equivalent of 25–50 % of the wet mass of the insect on a single oviduct. Furthermore, the continuous perfusion system we used ensures that hormones that may be secreted by the tissues themselves will be removed. Other bioassay systems, in which recordings are made from semi-isolated oviducts in chambers which are not continuously perfused with buffer, risk exposing tissues to locally secreted hormones. Insect tissues retain elements of the nervous system and even intrinsic peptidergic endocrine cells in the midgut which, when isolated in an *in vitro* system, may release neurochemicals into the incubation chamber. For example, adding an isolated midgut/hindgut segment to an isolated foregut preparation of the cricket *Teleogryllus commodus* caused an increase in foregut contractile activity (Cooper and He, 1994). It is possible that the exogenously applied neurochemicals in our assay could release neurotransmitters from any intrinsic neurons remaining on the isolated tissues. These neurotransmitters might have short-term effects on the myogenic contractions observed in our experiments.

Digital data-acquisition enabled the use of a variety of statistical and analytical techniques. From digital records, we were able to determine the rate and amplitude at which tissues contracted and to compute the velocity of contractions and the proportion of a cycle devoted to contraction. By depicting graphically the dynamics of a contraction cycle, state-series plots provided additional insights into the physiology of these contractile systems.

#### Leucomyosuppressin

Our results with LMS confirm some reports of the suppressive effects of this peptide (Holman *et al.* 1986) and contradict others. In studies of *Stomoxys calcitrans*, a species of the relatively advanced dipteran suborder Cyclorhapa, Cook and Wagner (1992) found that LMS did not inhibit myogenic contractions of oviducts. Neuropeptides differing from leucomyosuppressin only at the amino-terminal residue have been isolated from two higher dipterans, *Drosophila melanogaster* and the grey housefly *Neobellieria bullata* (Nichols, 1992; Fonagy *et al.* 1992). The peptide from the grey housefly was detected with heterologous bioassays using *Leucophaea maderae* hindguts and was found to inhibit

contractions of hindguts and oviducts from this cockroach; the *Drosophila* peptide was detected with a radioimmunoassay. The effects of these peptides on fly tissues were not reported.

For *A. aegypti*, which belongs to the more primitive suborder Nematocera, treatment of oviduct tissues with LMS in the range  $10^{-12}$  to  $10^{-8} \text{ mol l}^{-1}$  decreased the amplitude of the contractions. Analysis of the velocity profiles in Fig. 3C,D indicates that LMS depresses the amplitude by decreasing the velocity of the contractions. It does not appear to alter the endogenous pattern characteristic of the oviduct. This is reflected in the traces of Fig. 3E,F, which show similar shapes, though the absolute size of the shapes is different. Furthermore, Fig. 4 shows that LMS does not change the proportion of time during which the oviduct is actually contracting.

Hindguts responded to LMS at  $10^{-6} \text{ mol l}^{-1}$ , a concentration four orders of magnitude greater than the threshold reported for LMS in its homologous system (Holman *et al.* 1986). This may indicate that a peptide exerting LMS-like activity in the mosquito has a structure different from that of the LMS we

used, so that a much higher concentration of the substance is needed to produce a response. A concentration threshold as high as that found in our experiments was reported for the response of the stable fly hindgut to LMS, as was the transient nature of the response (Cook *et al.* 1991). A transient response to LMS is also known from *Leucophaea maderae* (Cook *et al.* 1993).

In control and LMS treatments, the intercontraction intervals of *A. aegypti* hindgut tissues are similar. However, after contractions have restarted in the presence of LMS, the hindgut tissues show more regularity in their contraction cycle. The state-series plots (Fig. 6E,F) show that, after contractions recover from LMS, the outline of the hindgut contraction cycle is simpler, indicative of a greater periodicity in the contractions.

#### Serotonin and octopamine

These neurochemicals produced identical physiological responses at the same dosages; only the response to serotonin will be discussed in detail.

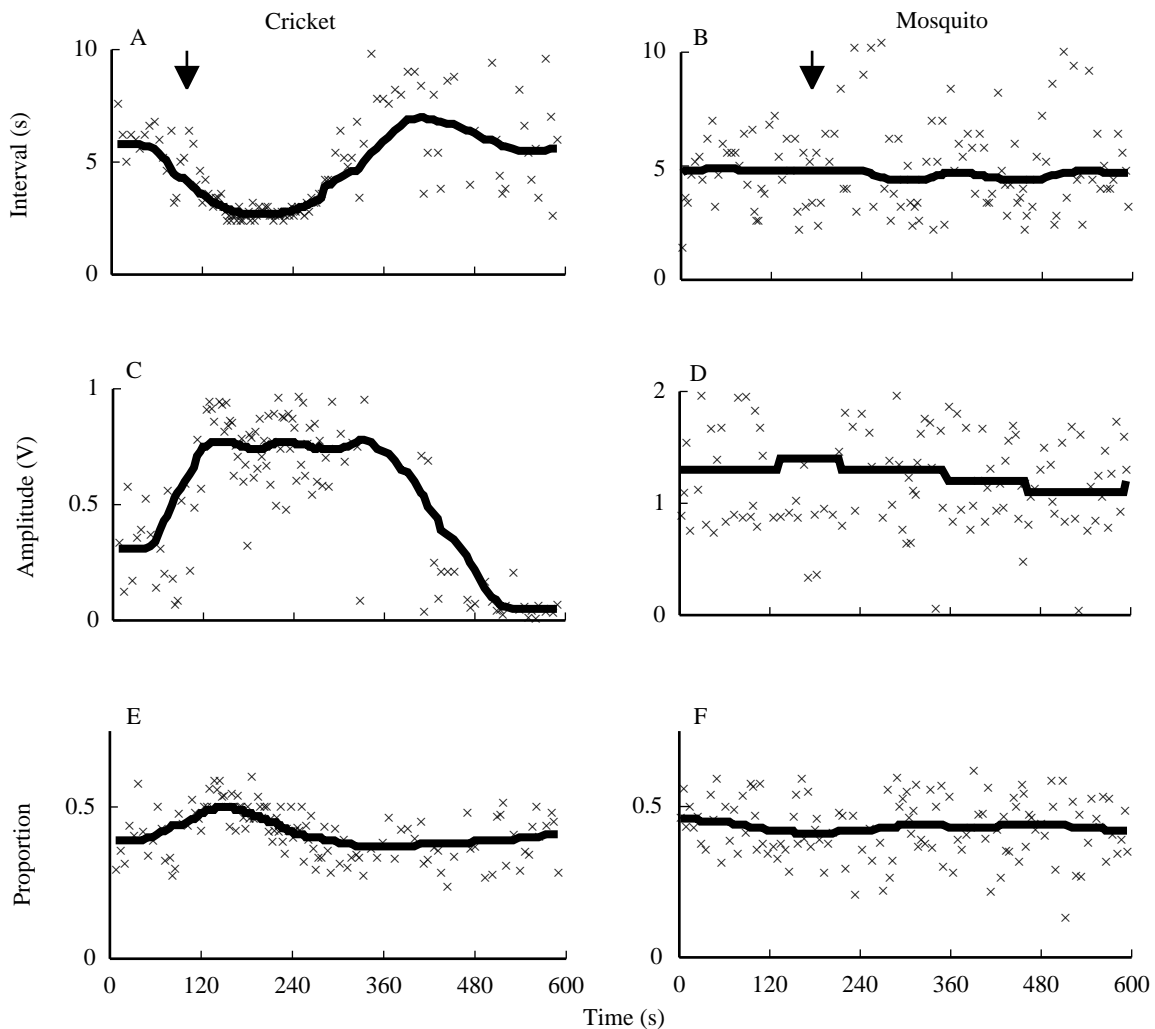


Fig. 12. Analysis of recordings in Fig. 11. Arrows indicate addition of  $10^{-8} \text{ mol l}^{-1}$  proctolin. (A,B) Effects of  $10^{-8} \text{ mol l}^{-1}$  proctolin on the intercontraction intervals of mosquito and cricket hindguts. (C,D) Amplitude of hindgut contractions. (E,F) Proportion of each contraction cycle devoted to contraction.

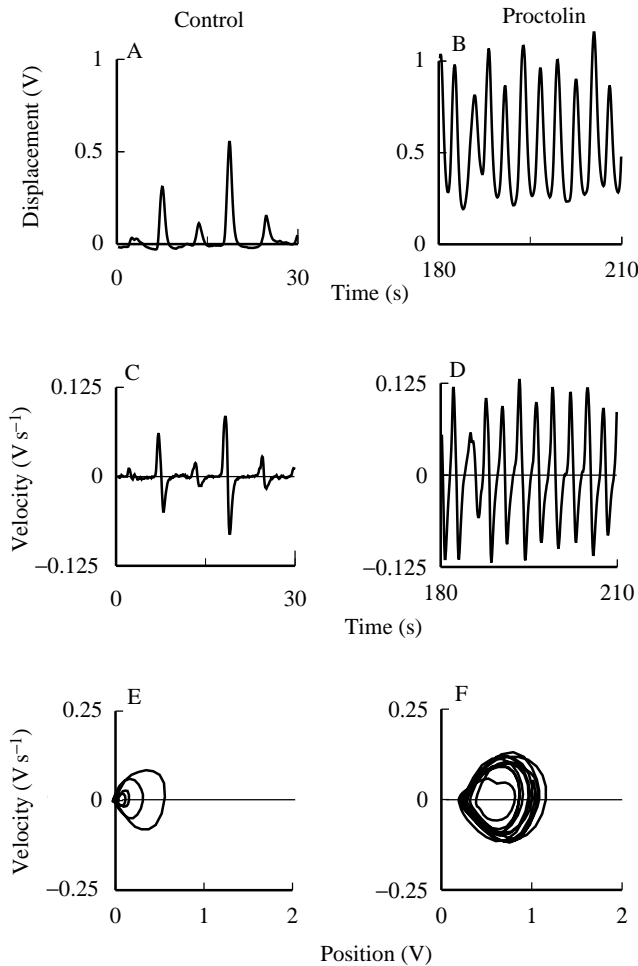


Fig. 13. Detailed analysis of ranges shown in Fig. 11B. (A,B) Amplitude response of cricket hindgut to proctolin. (C,D) Velocity profiles. (E,F) State-series analysis.

Serotonin increases the rate at which oviducts contract. Plots of intercontraction intervals and amplitude, shown in Fig. 7B,C show opposite trends. In the control buffer, there is a broad scatter of contraction intervals, while the amplitude of the contractions falls into a narrowly defined range. Treatment with  $10^{-8} \text{ mol l}^{-1}$  serotonin reverses these distributions. Contraction intervals decrease and fall into a narrowly defined range, while the range of amplitudes increases.

Compared with the oviduct in buffer (Fig. 8A), the contractions become more complex after serotonin treatment (Fig. 8B). The uniformity of the traces of velocity (Fig. 8D) and the state-series analysis (Fig. 8F) suggest that serotonin is not altering the underlying pattern of contraction of the oviduct. It appears to increase the frequency at which the endogenously generated pattern is repeated. The pattern seen in the state-series analysis (Fig. 8F) is produced because the tissue starts a new contraction before it has relaxed completely.

Oviducts of *Stomoxys calcitrans* respond differently to octopamine. In the same range of concentrations used in our experiments, octopamine inhibited contractions of stable fly oviducts (Cook and Wagner, 1992).

The response of oviducts to serotonin and octopamine has *in vivo* significance. During oocyte maturation, increased contractions of the oviduct and ovaries may be needed to ensure that developing oocytes are bathed in nutrient fluids. The nervous system of the mosquito *Aedes triseriatus* possesses serotonin and octopamine (Novak, 1992), and in other dipterans the distribution of serotonin has also been described (Cantera and Carlberg, 1988; Vallés and White, 1988).

Serotonin and octopamine had striking effects on the contractions of the *A. aegypti* hindgut. At  $10^{-6} \text{ mol l}^{-1}$ , both transformed the pattern from irregular waves of peristalsis to consistent contractions (serotonin, Figs 9, 10). Microscopic observations revealed that there were two components to the serotonin response: cessation of normal peristalsis and induction of regular, rapid contraction of the most anterior portion of the hindgut, just posterior to the insertion of the Malpighian tubules. The inhibition of peristalsis by serotonin has also been reported in the Colorado potato beetle *Leptinotarsa decemlineata*, but the contraction assay employed did not include the anteriormost region of the hindgut (van Haeften *et al.* 1993).

Serotonin amplifies the rate of secretion of Malpighian tubules in *A. aegypti*, increasing the volume of fluid entering the hindgut (Veenstra, 1988). We speculate that there is a link between this diuretic response to serotonin and its action on the hindgut. When hemolymph serotonin or octopamine titers are high, secretion from the Malpighian tubules into the hindgut increases. Peristaltic movements down the entire hindgut, which might generate back pressure on the tubules or push fluid into the midgut (which lacks the capacity to absorb water), are suppressed. The anterior portion of the hindgut remains active, physically pumping the fluid into the quiescent posterior region. As serotonin levels drop, peristalsis resumes, moving the fluid into the rectum where water is reabsorbed.

#### Proctolin

Repeated experiments failed to demonstrate that proctolin induced any detectable biological activity in *A. aegypti* oviduct and hindgut contraction assays. This is in contrast to the effect of this neurochemical on stable fly oviducts, which were stimulated by proctolin in the range  $10^{-9}$  to  $10^{-13} \text{ mol l}^{-1}$  (Cook and Wagner, 1992). Stable fly hindguts were stimulated by proctolin at concentrations ranging between  $10^{-15}$  and  $10^{-10} \text{ mol l}^{-1}$  (Cook *et al.* 1991). Cricket hindguts, treated with the same proctolin, responded in the expected manner. This provided a positive control for the mosquito experiments. Given the broad range of insect species (Orchard *et al.* 1989), including three dipterans (Cook and Meola, 1978; Irving and Miller, 1980; Cook *et al.* 1991; Cook and Wagner, 1992), in which proctolin has been shown to stimulate muscle contraction, the lack of response of *A. aegypti* is puzzling. There are only two reports on the presence of proctolin-like factors in Diptera: one by heterologous bioassay of whole-body extracts of adult and larval houseflies (*Musca domestica*; Brown, 1977) and the

other by immunocytochemistry in the abdominal ganglia and hindgut neurons (Nässel *et al.* 1989).

#### *Aedes Head Peptide I*

The total lack of response to Aea-HP-I by both muscle systems was quite unexpected, given its widespread distribution in the nervous and midgut endocrine system of *A. aegypti* (Brown and Lea, 1990) and its presence in the hemolymph, both before and after a blood meal (Brown *et al.* 1994). In sugar-fed mosquito females, at least, this peptide had no endocrine effects on these tissues. In addition, these results indicate that there is no neurotransmitter role for the peptide, an observation supported by the absence of any Aea-HP-I-containing axons on either the hindgut or the oviduct (M. R. Brown, unpublished observations).

#### *State-series analysis*

Physiological phenomena, such as muscle contractions, are usually reported as a function of time. The problem is that endogenously generated muscle contraction rhythms, or any rhythms of any tissue, may be bounded by variables other than time, with the result that a time-series plot (i.e. chart recorder trace) looks noisy or is simply difficult to interpret. The science of nonlinear dynamics, colloquially known as chaos theory, provides alternative methods of exploring experimental data.

Based on variables intrinsic to the tissue, state-series analysis offers a powerful method of examining muscle dynamics. A complex dynamic system can be plotted as a two-dimensional trace, with the result that physiological responses which might be independent of, or obscured by, the time domain become apparent. Application of techniques of chaos theory to relatively simple insect contractile systems indicates that this approach may be valuable in other physiological systems. The computational methods are straightforward and can be conducted with common spreadsheet or statistical software. The plots reveal trends which might be obscure when the data are presented as a function of time.

We applied state-series analysis by plotting the velocity of a contraction at a discrete position against the same position. Position corresponds to the transducer output; we calculated the velocity of the contraction by determining the rate of change of the voltage between successive time points. This produced diagrams which resemble work loops drawn for other muscle systems (Curtin and Woledge, 1993).

LMS effects on the contraction dynamics of *A. aegypti* oviducts reinforce interpretations made from time-series analysis of displacement and velocity. Following LMS treatment, the area outlined by the contracting oviduct is less than that of the control, but the shape of the contraction cycle is similar, indicating that the contractions are periodic. This contrasts with the state-series plot of the hindgut contractions which, under control conditions (Fig. 6A), show little regularity until after they have restarted in the presence of  $10^{-6} \text{ mol l}^{-1}$  LMS.

The effects of serotonin on oviduct contractions emphasize the power of state-series analysis. Under control conditions, the

tissue contracts spontaneously with great regularity, as is evident in the two time-series plots as well as the state-series plot (Fig. 8A,C,E). Serotonin causes the tissue to contract erratically (Figs 7A, 8B). But the state-series analysis (Fig. 8F) shows that the contraction dynamics outline a regular pattern not evident in the chart recorder trace.

#### *Common-sense application of chaos in physiological systems*

The most striking physiological finding of our research was the complete restructuring of the dynamics of hindgut contractions in response to  $10^{-6} \text{ mol l}^{-1}$  serotonin (Fig. 9). This tissue moved from a state of irregular peristaltic contractions to one displaying great regularity. However, watching the hindgut made it clear that different regions of this tissue responded differently to the serotonin; the anteriormost region was stimulated, while contractions in the remainder of the hindgut were inhibited. This response, which may be critical for understanding diuresis after a blood meal, would not have been evident from state-series analysis alone. Combining observational and transducer data allowed us to develop a full picture of the physiology of this tissue.

Our work has shown that chaos theory can be applied easily to physiological (and other) systems in which digital data streams are available. The techniques of state-series analysis will be a valuable adjunct to classical physiological investigations.

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