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Enhanced haemolymph circulation by insect ventral nerve cord: hormonal control by *Pseudaletia unipuncta* allatotropin and serotonin

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Summary

The ventral diaphragm (VD) in many insects is a muscular membrane that essentially partitions a perineural sinus from the rest of the abdomen. In the true armyworm moth *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) we describe how the VD is characterized by a series of aliform muscles inserted into a tissue matrix that is fused to the dorsal surface of the ventral nerve cord (VNC) itself. Because of this arrangement, the abdominal VNC can attain high rates of lateral oscillation, and is capable of directing haemolymph flow. We have previously demonstrated *Manduca sexta* allatotropin (Manse-AT)-like immunoreactivity throughout the central nervous system (CNS) in *P. unipuncta*, and that both Manse-AT and serotonin (5-HT) are dose-dependent stimulators of the dorsal vessel. Here we describe both Manse-AT- and 5-HT-like immunoreactivity associated with the VD. Furthermore, both Manse-AT and 5-HT are dose-dependent stimulators of the rates of VNC oscillation, and together are capable of maintaining highly elevated rates of VNC oscillation for extended periods of time. These data indicate that both the dorsal vessel and the VD/VNC are similarly modulated by both Manse-AT and 5-HT, and that VNC oscillations play a more active role in overall haemolymph circulation than previously recognized.

Key words: allatotropin, haemolymph circulation, *Manduca sexta*, *Pseudaletia unipuncta*, serotonin, ventral diaphragm, ventral nerve cord.

Introduction

The open circulatory system of arthropods may appear simple when compared to the vertebrate design. However, the unique morphologies of many insects has led to the development of specialized structures, such as the accessory pulsatile organs associated with the antennae, legs and wings (Miller, 1985; Pass, 1998, 2000), which work in concert with the dorsal vessel to ensure effective haemolymph circulation into these appendages. In addition, rhythmic muscle contractions causing pulsations of the abdominal cuticle have also been implicated in facilitating circulation (Sláma, 1984, 2000). Furthermore, in many insects the ventral diaphragm (VD), a continuous muscular membrane that forms a perineural sinus separate from the rest of the abdomen, plays a somewhat limited role in abdominal haemolymph circulation (Snodgrass, 1935; Jones, 1954; Guthrie, 1962; Richards, 1963; Heinrich, 1971; Peters, 1977).

In most Lepidoptera, the VD has developed into a unique structure, originally named the Cord of Leydig after the researcher who made the original observations (Leydig, 1862; cited in Brocher, 1920). Instead of a continuous sheet lying dorsally to, yet separated from, the ventral nerve cord (VNC), the lepidopteran VD consists of a discontinuous arrangement of fine aliform muscle fibres originating ventrolaterally on the abdominal sternites, and inserting onto a connective tissue matrix on the abdominal midline (Richards, 1963; Ashhurst and Richards, 1964a; Hessel, 1969; Chapman, 1971; Kristensen and Nielsen, 1980). This tissue matrix, comprising primarily collagen (Ashhurst and Richards, 1964a,b; Ashhurst, 1968), is fused to the top of and/or partially surrounds the VNC. Consequently in Lepidoptera, contractions of the VD musculature can cause lateral oscillations of the VNC itself, and not just the movement of the VD as seen in other insects. In the moths *Bombyx mori* and *Arctias luna*, the VNC was reported to oscillate at frequencies of approximately 80 and 120 cycles per minute (Gerould, 1938; Richards, 1963). In addition, data indicate that these contractions were able to specifically move haemolymph from the anterior to the posterior portion of the abdomen (Brocher, 1920; Hessel, 1969), suggesting that the function of the collagen matrix (and the associated musculature) was more than just providing a protective sheath for the VNC. Furthermore, Brocher (1920) noted that the frequency of these lateral movements changed, being low when the insect was at rest and high when the insect was ‘excited’. Since these initial observations, however, we are not aware of any research that has been undertaken to elucidate any physiological mechanisms underlying these changes in frequency in the Lepidoptera.

The true armyworm *Pseudaletia unipuncta* has been used as a model system to study the role of juvenile hormone (JH) in the pheromone-mediated reproductive biology of moths undertaking seasonal migrations in response to habitat deterioration (Delisle et al., 1987; McNeil, 1987; Cusson et al., 1990, 1993, 1994a,b; McNeil et al., 1994, 1995, 1996, 2000). We have cloned the gene for the neuropeptide, *Manduca sexta* allatotropin (Manse-AT), in *P. unipuncta* (Truesdell et al., 2000); the gene was originally identified and characterized in the moth *M. sexta* based on its ability to stimulate *in vitro* JH biosynthesis (Kataoka et al., 1989). Both the expression of this gene, and Manse-AT-like immunoreactivity, were shown to be distributed throughout the brain and abdominal ganglia of the armyworm (Truesdell et al., 2000). The pleiotropic nature of this peptide has been reported for several moth species and non-lepidopterans; it is not only capable of stimulating *in vitro* JH biosynthesis (Audsley et al., 1999, 2000; Oeh et al., 2000; Rachinsky et al., 2000; Tu et al., 2001), but also acts as a cardioacceleratory agent (Veenstra et al., 1994; Rudwall et al., 2000) as well as inhibiting midgut ion transport (Lee et al., 1998). A general model, including the possible pleiotropic effects of Manse-AT on the reproduction and migration of *P. unipuncta*, was proposed (McNeil and Tobe, 2001) and subsequent work demonstrated that Manse-AT affects both JH biosynthesis and cardioacceleration in true armyworm adults (Koladich et al., 2002). Furthermore, it was shown that serotonin (5-hydroxytryptamine, 5-HT) is a potent stimulator of heart rate in adult *P. unipuncta* (Koladich et al., 2002), as seen in *M. sexta* (Prier et al., 1994) and several other invertebrates (Chiang et al., 1992; Fox and Lloyd, 1997; Saver et al., 1999; Zornik et al., 1999). Although 5-HT affected the intensity and duration of the cardioacceleration significantly more than Manse-AT, a clear synergistic interaction was seen when the dorsal vessel was stimulated simultaneously with both compounds (Koladich et al., 2002).

The true armyworm *P. unipuncta* is a powerful flying insect, and has an efficient circulatory system that is essential for long-distance migrations in search of suitable habitats. In the present paper, we extend our investigation of the roles of both Manse-AT and 5-HT in haemolymph circulation in *P. unipuncta* by analyzing their effects on the VD. We suggest that this structure plays a more active role in the circulatory system than previously thought, and is essential to the animal in terms of carbohydrate and lipid mobilization, fight energetics and thermoregulation.

Materials and methods

Animals

Day-3 adult virgin male and female *Pseudaletia unipuncta* (Haworth) were utilized in all experiments, taken from colonies established using field-collected adults during the summer of 2000 in Quebec, Canada. Larvae were reared on artificial pinto bean diet (Shorey and Hale, 1965), whereas the adult sexes were held in separate cages until needed and provided with a solution of 8% sucrose *ad libitum*. All animals were maintained at 25±0.5°C, 65±5% RH under a 16 h:8 h light:dark photoperiod.

Reagents

All chemicals were supplied by Sigma (St Louis, MO, USA), except for goat anti-mouse secondary antisera (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA); Manse-AT was custom-synthesized and purified by high performance liquid chromatography (to >90% purity) by Research Genetics (Huntsville, AL, USA).

Anatomy and ventral nerve cord function

To obtain still images of the VNC and VD structures, entire male and female abdomens were fixed with 2.5% glutaraldehyde in 0.1 mol l^{-1} Sorenson's phosphate buffer, pH 7.2, for 12–14 days. Approximately 500 μm of the extreme anterior and posterior ends of the abdomen (primarily cuticle) were removed prior to fixation to facilitate penetration of the glutaraldehyde. For cross-sectional images, multiple transverse sections were made with a scalpel, and the sections stained for 30 s with 0.1% Methylene Blue and 7.5 mmol l^{-1} ascorbic acid in ddH_2O to enhance contrast. Alternatively, intact VNC and associated VD musculature were carefully dissected out of fixed abdomens and stained for 30 s with 0.1% Methylene Blue and 7.5 mmol l^{-1} ascorbic acid in ddH_2O followed by staining for 30 s with 0.1% Neutral Red in ddH_2O. After staining, both transverse sections and intact VNC/VD tissues were thoroughly rinsed in phosphate-buffered saline (PBS; 10 mmol l^{-1} phosphate buffer in 0.9% NaCl, pH 7.2). All still images were immediately captured on an Olympus C4040 Zoom 4.1 Megapixel digital camera mounted on an Olympus BX60 System light microscope.

To acquire images of the oscillating VNC, live animals were pinned dorsal side up with their head, legs, wings and removed. The dorsal cuticle was removed along with the dorsal vessel. The fat body, gut and reproductive organs were carefully dissected away under a modified Weever's insect saline (7.0 mmol l^{-1} NaCl, 34.0 mmol l^{-1} KCl, 16.0 mmol l^{-1} $MgCl_2$, 4.0 mmol l^{-1} $CaCl_2$, 184.0 mmol l^{-1} glucose, pH 6.8) (Lehman et al., 1993), thus exposing the oscillating VNC. Tissues were then flooded with 0.1% Methylene Blue and 7.5 mmol l^{-1} ascorbic acid in ddH_2O for 20 s, followed by thorough rinsing in modified Weever's saline. The movement of the VNC was then recorded in the form of digital video using a Panasonic GP-KR412 charge-coupled device mounted on a Zeiss dissecting light microscope. Still images were then captured on a computer from sequential frames of video.

To acquire images of the circulatory abilities of the oscillating VNC, live animals were dissected as described above. However, the fat body, gut and reproductive organs were left intact. Dye was then introduced with a fine syringe into the intact thorax, or into the exposed fat body of either the anterior or posterior abdomen. Sequential images of the resultant movement of dye were generated as described above.

Whole-mount immunocytochemistry

All steps were performed at room temperature unless

otherwise stated. Animals were anaesthetized by chilling on ice. Brains and VNC were dissected under ice-cold modified Weever's saline, and placed immediately in ice-cold fixative (2% paraformaldehyde in Millonig's buffer). Tissues were fixed overnight at 4°C and rinsed thoroughly in PBS. Tissues were then incubated for 2 h in PBS containing 4% Triton X-100, either 10% normal goat serum (NGS; for Manse-AT assays) or 10% normal sheep serum (NSS; for 5-HT assays) and 2% protease-free bovine serum albumin (BSA), followed by 30 min in PBS containing 3% skimmed milk powder. The 4% Triton X-100/10% NGS/NSS, and skimmed milk incubations, were performed to increase perineurium permeability, enhance antibody penetration and reduce non-specific binding of antibodies. Primary antibodies were also pre-incubated for 24 h at 4°C in PBS containing 0.4% Triton X-100, 2% BSA and 2% NGS (Manse-AT) or 2% NSS (5-HT) to occupy non-specific antigenic sites.

To identify Manse-AT-like immunoreactivity, tissues were incubated in a murine monoclonal antibody diluted 1:800 in PBS containing 0.4% Triton X-100, 2% NGS and 2% BSA for approximately 5 days at 4°C. To identify 5-HT-like immunoreactivity, tissues were incubated in a rabbit polyclonal antibody diluted 1:800 in PBS containing 0.4% Triton X-100, 2% NSS and 2% BSA for approximately 2 days at 4°C before being washed overnight in PBS containing 0.2% Triton X-100 at 4°C. Manse-AT-like immunoreactivity was visualized by incubating the tissues for 18 h at 4°C in fluoroscein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antiserum diluted 1:200 in PBS containing 0.4% Triton X-100, 10% NGS and 2% BSA. 5-HT-like immunoreactivity was visualized in the same manner using Cy-3-conjugated sheep anti-rabbit secondary antiserum in 10% NSS. All tissues were then rinsed overnight at 4°C in PBS containing 0.2% Triton X-100, cleared and mounted on slides in 5% *n*-propyl gallate in 80% glycerol (pH 7.3). Images were captured using a Zeiss LSM 510 laser scanning confocal microscope employing an argon laser/FITC filter set (Manse-AT) and helium–neon laser/Cy-3 filter set (5-HT).

Liquid-phase pre-adsorption of the Manse-AT primary antiserum with 10 μmol l^{-1} synthetic Manse-AT, or the 5-HT primary antiserum with 10 μmol l^{-1} 5-HT, abolished signals in all immunopositive tissues. Furthermore, no immunoreactivity was detected in either assay when either the primary or the secondary antibody incubation was omitted from the procedure.

Ventral nerve cord assays

Live animals were pinned dorsal side up into a Sylgard dish with their head, legs and wings removed. Dorsal midline incisions in the abdomen were made, and the heart, gut and reproductive organs were removed. The VNC was exposed and kept flooded with modified Weever's saline. The electrodes from an impedance converter (UFI Instruments model 2991; Morro Bay, CA, USA) were positioned approximately 3 mm apart with the VNC centered between them. The resultant signal was captured on a chart-recorder (Linear Instruments Corp. model 1200; Reno, NV, USA). All electrophysiology equipment was appropriately grounded and shielded in a Faraday cage. These semi-isolated VNC preparations were bathed in 0.5 ml of modified Weever's saline for a minimum of 20 min (with changes every 5 min), and only those preparations that maintained a stable rate and did not respond to the changes in saline, were used. Different concentrations of synthetic Manse-AT, 5-HT or both chemicals, dissolved in modified Weever's saline, were then manually applied with 3×0.5 ml washes. Changes in the rate of VNC oscillation were quantified by comparing the average rate (beats min^{-1}) for the 2 min immediately prior to application of a compound with the rate during the 1 min immediately after application for dose–response assays, and for every subsequent minute for an overall period of 20 min in the case of time-course experiments. Because of the nature of the signal detected by the impedance converter, only changes in frequency, not amplitude, could be quantified accurately. A minimum of five animals was used per data point.

Results

Anatomy and time-lapse photography

Staining of the VNC, VD and surrounding tissue demonstrated the presence of fine aliform muscles, which originated ventrolaterally from discrete points on the abdominal sternites. These muscles inserted into a connective tissue matrix, which itself lay fused to the dorsal side of the VNC (Fig. 1A–D). From their origins, the muscles fanned out to form insertion points along the entire length of the connective tissue matrix. Although there is a continuous network of muscle fibres, they did not form a continuous membrane sheet, as has been reported for many other insect species. The VD musculature and connective tissue matrix was present on the entire abdominal VNC, but appeared to be absent in the thorax. The muscles seemed to not have any identifiable segmental pattern, and were somewhat irregularly ordered (Fig. 1C,D).

The abdomens of adult virgin *P. unipuncta* were densely packed with fat body. However, surrounding the VNC, there was a perineural sinus approximately 300–500 μm wide, essentially forming a hollow tube that runs longitudinally along the entire abdomen and inside which the VNC oscillates (Fig. 1B). Although the VNC is only approximately 80 μm wide, it was occasionally observed to oscillate with an amplitude of approximately 200 μm from the ventral midline (Fig. 2).

When dye was introduced into either the thorax or anterior abdomen of live animals from which the dorsal cuticle had been removed, it reappeared through the fat body in the region dorsal to the terminal abdominal ganglion (TAG; Fig. 3) within approximately 0.5 s, which was a shorter time period than would be possible by diffusion alone. No dye was ever observed moving laterally through the fat body or elsewhere along the length of the VNC. In addition, following the introduction of dye to the posterior end of the abdomen, it

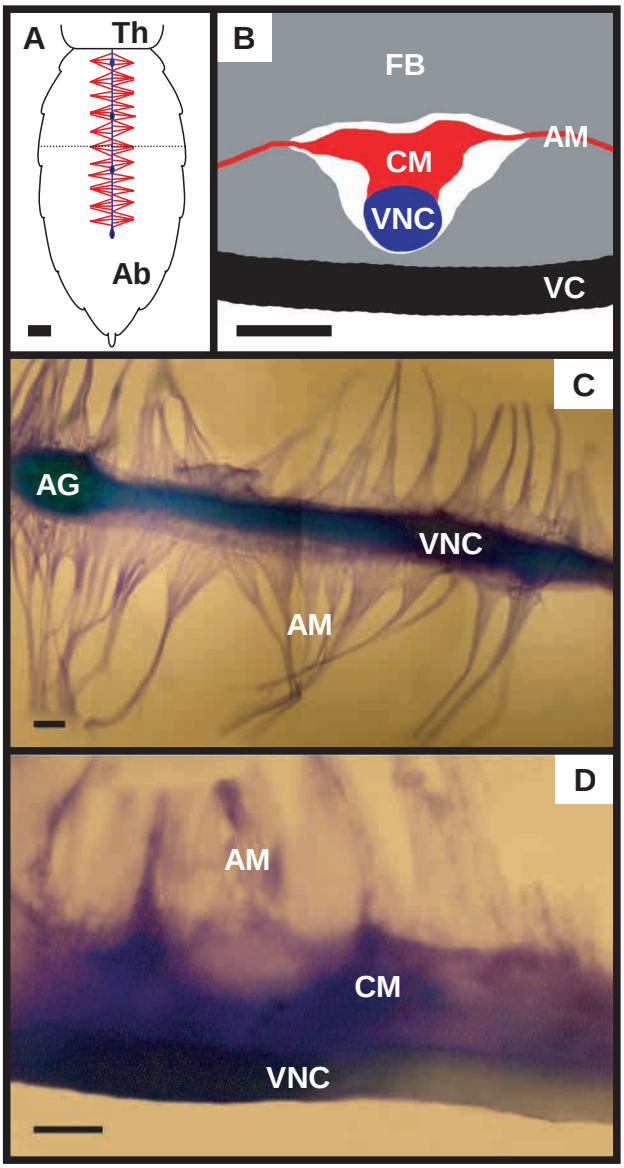

Fig. 1. Anatomy of the ventral nerve cord (VNC) and ventral diaphragm (VD) in adult male and female *P. unipuncta*. (A) Schematic representation of the abdomen (Ab), indicating the relative positioning of the VNC (blue) and VD (red) in dorsal view. Th, thorax. Note that due to scaling constraints, the VNC and VD are represented approximately twice as wide as they are *in situ*. (B) Colour representation of a transverse section through the adult abdomen, taken at the position of the dotted line in A. (C,D) Dorsal and lateral views of the VNC (blue-grey) and VD, comprising the collagen matrix (CM) and associated alary muscles (AM) (violet). AG, abdominal ganglia; FB, fat body; VC, cuticle. Scale bars, 1 mm (A); 100 μm (B–D).

remained at the site of introduction and was not detected in either the anterior abdomen or thorax.

Manduca sexta *allatotropin-like immunocytochemistry*

Immunocytochemistry of the VNC of day-3 adult male and female *P. unipuncta* indicated the presence of three pairs of soma arranged medially within the abdominal ganglia, and

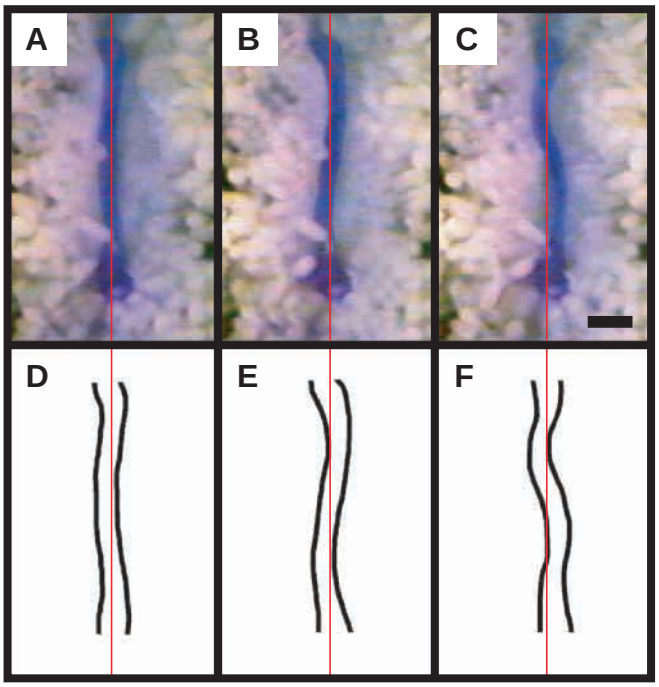

Fig. 2. (A–C) Time-lapse photo sequence of the oscillating ventral nerve cord (VNC) of adult *P. unipuncta* moths. The VNC was stained to enhance contrast with the surrounding fat body. (D–F) Outline of VNC from the panels A–C, respectively. The red lines indicate the ventral mid-line of the abdomen. Scale bar, 200 μm.

axons within the VNC connectives that demonstrated Manse-AT-like immunoreactivity. Furthermore, there was an extensive network of Manse-AT-like immunoreactive fibres running the entire length of the abdominal nerve cord, but not in the TAG (Fig. 4A). These immunoreactive axons exit the abdominal ganglia laterally and immediately branch dorsally, sending both contra- and ipsilateral projections anterior and posterior along the dorsal surface of the tissue matrix fused to the VNC. These axons appear to possess extensive sites of release. No differences were noted between the sexes.

Serotonin-like immunocytochemistry

2–3 ventrolateral pairs of 5-HT-like immunoreactive cells were located posterior in each of the abdominal ganglia, which contributed to the immunoreactivity detected in axons running within the VNC connectives, as well as those exiting laterally from the VNC. An extensive network of 5-HT-like immunoreactive axons was detected along the entire length of the tissue matrix associated with the VNC, but was absent from the TAG, similar to the pattern seen with Manse-AT-like immunoreactivity. These axons, which also appeared to possess numerous release sites, were also situated dorsally and frequently ran both within and along the surface of the matrix (Fig. 4B). Positive 5-HT-like soma were also detected in several sites in the brain, corpora cardiaca and TAG of day-3 adult males and females (data not shown). No differences were noted between the sexes.

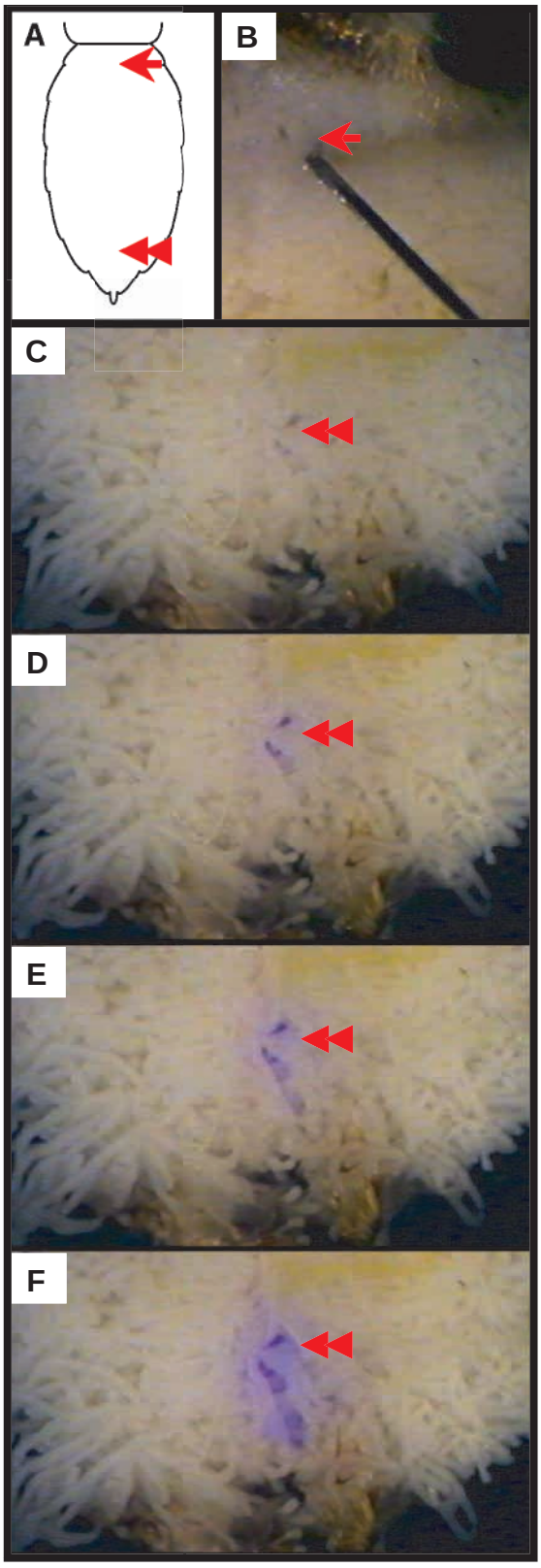

Fig. 3. Time-lapse photo sequence of active movement of dye resultant from ventral nerve cord (VNC) oscillations in *P. unipuncta* adult moths. (A) Diagram of abdomen in dorsal view indicating the site of dye introduction in the anterior abdomen (arrow) and the region where the dye reappears in the posterior (double arrowheads). (B) Insertion of the syringe through the copious amounts of fat body at the site of injection (arrow). (C–F) Photographic sequence at 0.1 s intervals after injection showing the appearance of dye in the region of the posterior abdomen dorsal to the terminal abdominal ganglion.

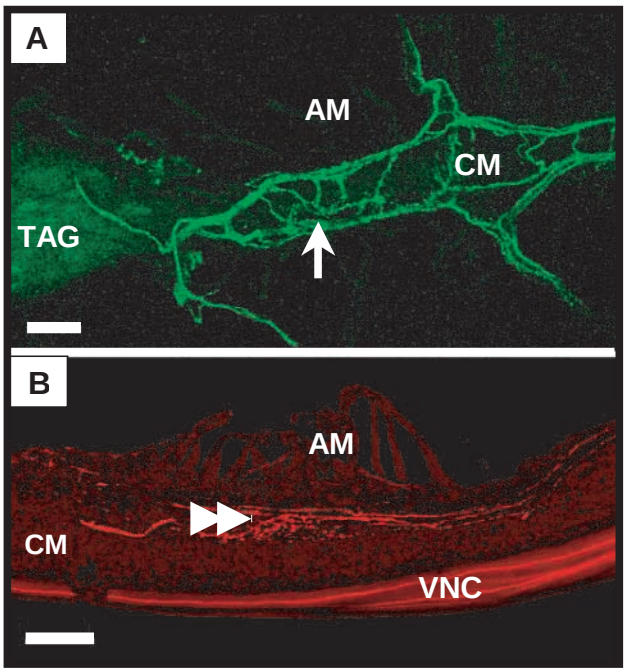

Fig. 4. Immunoreactivity (IR) associated with the ventral nerve cord (VNC) and ventral diaphragm (VD) of a day-3 adult *P. unipuncta* female. (A) Manse-AT-like immunoreactivity, dorsal view; (B) 5-HT-like immunoreactivity, lateral view. Whereas Manse-AT-like immunoreactive nerves run primarily on the dorsal surface of the collagen matrix (arrow), 5-HT-like immunoreactivity is seen primarily associated with nerves within the collagen matrix itself (double arrowheads). AM, alary muscles; CM, collagen matrix; TAG, terminal abdominal ganglia; VNC, ventral nerve cord. Scale bars, 100 μm.

Stimulatory effects of allatotropin

The VNC of both sexes of *P. unipuncta* oscillate laterally at rate of approximately 135 cycles min^{-1} when resting. Treatment of the VNC of day-3 adult male and female armyworms with Manse-AT at 10^{-9}–10^{-5} mol l^{-1} resulted in positive dose-dependent responses in the rate of VNC oscillations in both sexes (Fig. 5). The EC_{50} values in males and females were comparable at 1.2×10^{-7} mol l^{-1} and 2.2×10^{-7} mol l^{-1}, respectively. Concentrations at or below 10^{-9} mol l^{-1} had essentially no significant stimulatory effect, whereas both sexes showed significant increases from the resting rate at 10^{-6} mol l^{-1} and above (*t*-test; $P<0.05$). Maximum levels of stimulation (approximately 25% above resting) reached a plateau at approximately 10^{-6} mol l^{-1}.

Stimulatory effects of serotonin

Serotonin also proved to be a potent stimulator of the rate

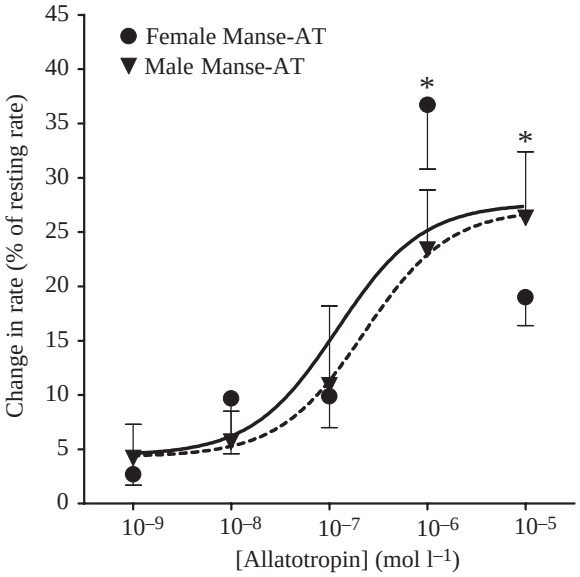

Fig. 5. The dose-responses of semi-isolated adult virgin day-3 male and female *P. unipuncta* ventral nerve cord (VNC) upon exposure to increasing concentrations of Manse-AT in saline. Data are expressed as percentage increase in the resting rate of oscillation and values are means ± S.E.M. (*N*=5 or more samples). *Significant increase from resting rate (P<0.05).

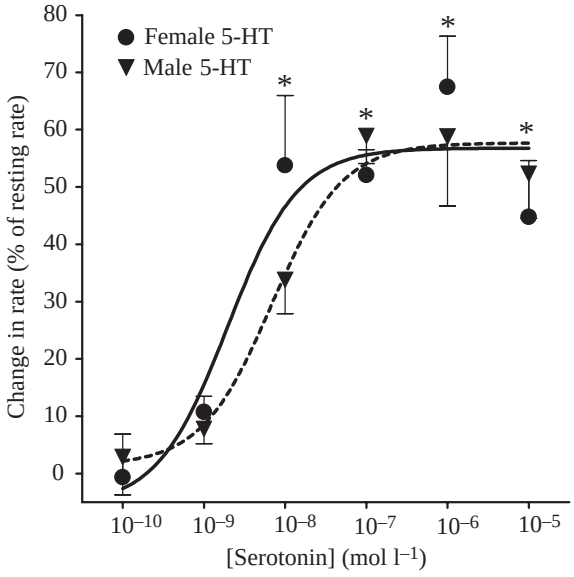

Fig. 6. The dose-responses of semi-isolated adult *P. unipuncta* virgin day-3 male and female ventral nerve cord (VNC) upon exposure to increasing concentrations of 5-HT in saline. Data are expressed as percentage increase in the resting rate of oscillation and values are means ± S.E.M. (*N*=5 or more samples). *Significant increase from resting rate (P<0.05).

of VNC oscillations in *P. unipuncta*, with day-3 males and females showing similar dose-responses at concentrations from 10^{-10} mol l^{-1} to 10^{-5} mol l^{-1} (Fig. 6). However, EC_{50} values for 5-HT at 6.8×10^{-9} mol l^{-1} and 2.0×10^{-9} mol l^{-1} for males and females, respectively, were several orders of magnitude lower than the EC_{50} values seen for Manse-AT. Accordingly, significant increases over resting rates are seen at 10^{-8} mol l^{-1} (*t*-test; P<0.05). In addition, 5-HT treatment resulted in maximum rates of oscillation >55% above the resting rate, which was more than twofold higher than the increase of approximately 25% seen with Manse-AT.

Allatotropin and serotonin time courses

Manse-AT and 5-HT, applied at concentrations close to their EC_{50} values (1×10^{-6} mol l^{-1} for Manse-AT; 1×10^{-8} mol l^{-1} for 5-HT), both evoked a rapid acceleration of the rate of VNC oscillations in day-3 adult females, but resulted in dramatically different temporal patterns (Fig. 7). Manse-AT treatment alone resulted in a significant increase in rate of oscillations within the first minute of application (*t*-test, P<0.05) but rates gradually returned to pre-exposure levels within 5 min. Subsequent applications of 10^{-6} mol l^{-1} Manse-AT at 2, 4 and 6 min after initial stimulation (t=0) resulted in a sustained significant level of stimulation for 7 min (*t*-test, P<0.05) but rates nonetheless returned to approximately pre-exposure levels in less than 3 min following the last treatment (Fig. 8).

Treatment with 5-HT alone also resulted in a dramatic, significant increase within the first minute. However, in contrast to the results from exposure to Manse-AT, rates remained significantly elevated above resting levels for the duration of the experiment (Fig. 7; *t*-test, P<0.05). Furthermore, 5-HT stimulation consistently resulted in levels of stimulation that were significantly higher than the Manse-AT treatment over the entire time course of the experiment (*t*-test; P<0.05).

Simultaneous treatment of day-3 adult females with both Manse-AT and 5-HT at approximately their EC_{50} values displayed a temporal pattern similar to, but slightly reduced from treatment with 5-HT alone, and did not show any additive or synergistic effects (Fig. 7). However, whereas the slope of the line for 5-HT-treated VD from t=2 to 20 min showed a gradual decline that was significantly different from zero (slope=–0.3926; F=49.81, P<0.001), the slope of the line for Manse-AT+5-HT treated VD maintained a constant, accelerated rate that was not different from zero (slope=–0.0541; F=0.2824, P=0.6179), indicating that there was no decline over the period examined. In addition, the slopes of these two lines are significantly different from each other (ANCOVA; F=8.510, P=0.015).

Discussion

Manse-AT and 5-HT not only affect the rate of contraction of the dorsal vessel (Koladich et al., 2002) but also modulate contractions of the VD. Furthermore, whereas exposure of the VD to either of these hormones alone showed gradual decreases in the rate of oscillation over time, exposure to both Manse-AT and 5-HT simultaneously produced a constant, elevated rate of oscillation that, which after the initial surge from t=0–2 min, did not decrease over the duration of our

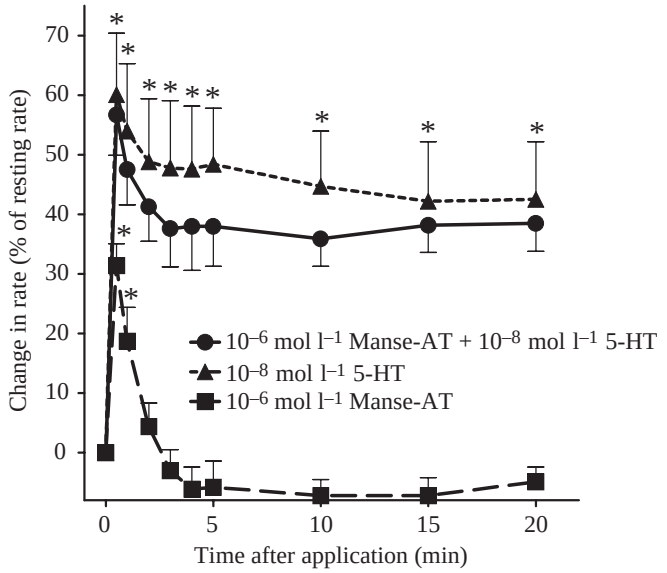

Fig. 7. Temporal changes in the rate of oscillation of the ventral nerve cord (VNC) of *P. unipuncta* following application of Manse-AT or 5-HT in saline, independently or concurrently, at *t*=0 to semi-isolated VNC of adult virgin day-3 females at concentrations approximating their EC_{50} values. Data are expressed as percentage increase in the resting rate and values are means ± S.E.M. (*N*=5 or more samples). *Significant increase from resting rate ($P<0.05$).

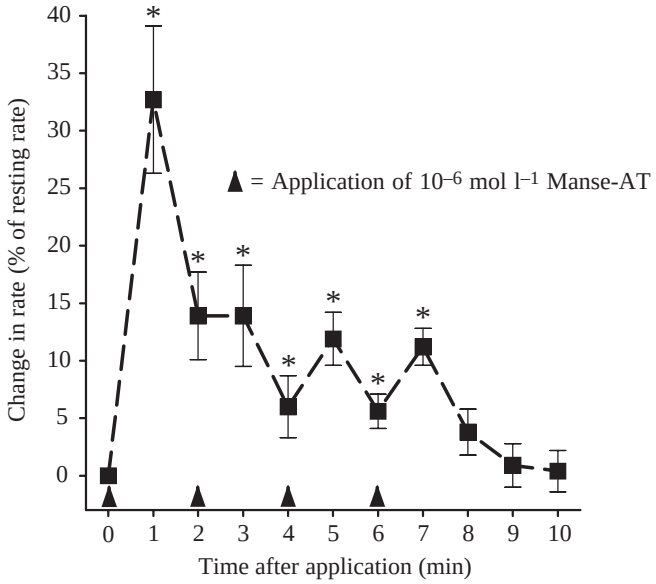

Fig. 8. Effects of repeated exposure of Manse-AT on the rate of oscillation of semi-isolated adult *P. unipuncta* virgin day-3 female VNC. Manse-AT was serially applied at approximately the EC_{50} value at 0, 2, 4 and 6 min. Data are expressed as percentage increase in resting rate and values are means ± S.E.M. (*N*=5 or more samples). *Significant increase from resting rate ($P<0.05$).

experiment. This time-stable response is consistent with the one observed when the dorsal vessel was concurrently exposed to Manse-AT and 5-HT (Koladich et al., 2002). These data indicate that both hormones are involved in the modulation of these two circulatory structures, and suggest a possible coordination of processes between the heart rate and oscillations of VNC. Synchrony between these different structures would provide an effective means of circulation, with the dorsal vessel and VD moving blood to the anterior and posterior sections of the abdomen, respectively.

These findings certainly suggest that, at least in *P. unipuncta*, the VD may play a much more active role in the circulation of haemolymph than previously thought. There are several ways in which this would be of particular importance for species such as the true armyworm, which has very large fat reserves and uses powered flight during long-distance migration. Orchard et al. (1991) have shown that in the true armyworm, haemolymph lipid levels, under the control of adipokinetic hormone, increased >150% above resting after 1 h of sustained flight. Thus, there would be considerable physiological benefit to an effective mobilization of energy sources from the abdomen to the flight muscles. Similarly, the efficient removal of metabolic waste from the thorax would ensure that their accumulation did not adversely affect muscle function. These two processes would be facilitated by the complimentary action of the dorsal vessel and the VD.

Another very important function of active haemolymph movement from the thorax to the abdomen is thermoregulation, as shown in a number of insects including dragonflies, bees and Lepidoptera (Heinrich, 1970a, 1974, 1976; Heinrich and Casey, 1978). Sustained flight generates considerable heat, and without an effective means of dissipating heat from the thorax, temperatures would soon rise to critical levels. Heinrich (1971) has clearly shown that, as thoracic temperature rises in the tobacco hornworm *M. sexta*, there is an increase in abdominal temperatures. In addition, it occurs sooner and reaches higher levels beneath the VD than in the dorsal section of the abdomen. Furthermore, heart rate increases with increasing thoracic temperatures and is regulated by neural mechanisms (Heinrich, 1970b). Thus, it is possible that a similar neural feedback exists with respect to the VD.

The presence of extensive immunoreactivity and numerous release sites for both Manse-AT and 5-HT along the length of the VNC suggests that secretion of either or both of these neurohormones into the haemolymph would occur quickly and efficiently. This would not only expedite the rapid movement of cardioactive compounds to the dorsal vessel, but the corelease of other neurohormones with either Manse-AT or 5-HT would ensure very rapid and efficient circulation to other parts of the body – an elegant solution for a moth with an abdomen densely packed with fat body.

Interestingly, the effects of Manse-AT on both the VD and dorsal vessel are very short-lived, even with repeated exposure (Koladich et al., 2002). In our time-course experiments, the VD only showed a significant response to Manse-AT with the initial exposure, and reapplication of peptide resulted in only minor increases in rates of VD oscillation (Fig. 8). This suggests rapid desensitization of Manse-AT receptors,

although it does not rule out the possibility that Manse-AT is rapidly degraded by peptidases released in response to the initial application of peptide. This may nevertheless have some bearing on the pleiotropic nature of the Manse-AT. *Manduca sexta*-AT is capable of stimulating JH biosynthesis in the corpora allata of adult *P. unipuncta* (Koladich et al., 2002), so it may be desirable to have only small quantities of this peptide released from the VNC *in vivo* to 'prime' the VD and/or dorsal vessel for exposure to 5-HT during migratory flight by sexually immature individuals. If larger amounts of Manse-AT were required for maintaining stimulated levels of the dorsal vessel and/or the VD, then untimely effects on JH biosynthesis might be result. This is significant, for while low levels of JH are required during migration, higher levels would induce premature sexual maturation and reproductive behaviour in adult male and female *P. unipuncta* (Rankin and Riddiford, 1978; Delisle et al., 1987; McNeil 1987; Cusson et al., 1990, 1994a,b; McNeil et al., 1994, 1996, 2000).

In conclusion, our data suggest that the VD plays an important role in fluid circulation of adult *P. unipuncta*. Both Manse-AT and 5-HT are critical hormonal elements that augment circulation by modulating the VD as well as the dorsal vessel, allowing enhanced transport of haemolymph, lipids, and metabolic and thermal wastes, very necessary for this long-distance migrant.

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