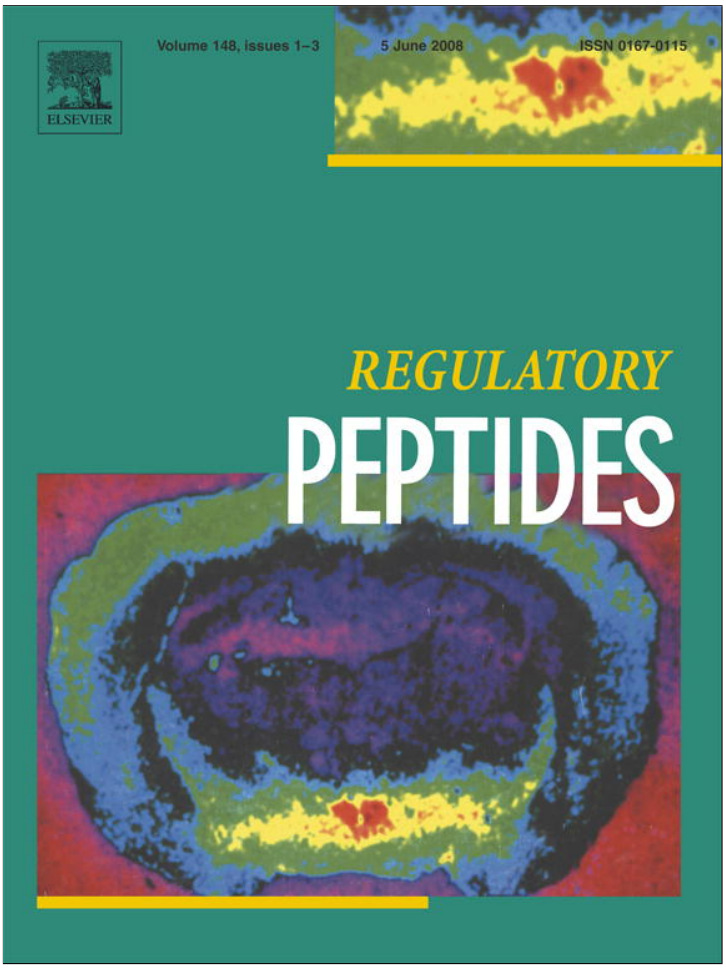

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Antifeeding properties of myosuppressin in a generalist phytophagous leafworm, *Spodoptera littoralis* (Boisduval)

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Abstract

Insect myosuppressins are a family of peptides with a characteristic HV/SFLRFamide carboxy terminus. They are expressed in brain, neurohemal organs, stomatogastric nervous system, and in midgut endocrine cells. From a functional point of view, myosuppressins inhibit contractions of different visceral muscles, stimulate certain skeletal muscles and activate enzyme secretion from the gut. Moreover, in the omnivorous cockroach *Blattella germanica*, myosuppressin inhibits food intake. Based on these results, we studied the antifeeding activity of myosuppressin in the phytophagous leafworm *Spodoptera littoralis*. Firstly, we isolated the cDNA corresponding to the *S. littoralis* myosuppressin precursor encoding the typical myosuppressin peptide of lepidopterans: pQDVVHSFLRFamide. Then, we determined the expression patterns (in terms of mRNA and peptide) of myosuppressin in brain and midgut, and peptide levels in the haemolymph. Myosuppressin patterns in the brain and haemolymph were similar, and symmetrical to that of food consumption, thus suggesting that myosuppressin might inhibit feeding in *S. littoralis*. Moreover, synthetic myosuppressin effectively inhibited food intake in non-choice antifeeding tests. Taken together, the obtained results point to the hypothesis that myosuppressin represses feeding in *S. littoralis*.

Keywords: Myosuppressin; Antifeeding; Leafworm; *Spodoptera littoralis*

1. Introduction

Neuropeptides are widespread signalling molecules in the nervous system, which display multiple functions and different mechanisms of action. Among neuropeptides, myosuppressins are especially abundant and have been described in diverse insect groups [1,2]. They share the same C-terminal–FLRFamide sequence, and were initially identified as a result of their capacity of inhibiting gut contractions in cockroaches. Later, this property has been demonstrated in a number of cockroach and locust species [3–7].

The myosuppressin gene or cDNA has been cloned and sequenced in the fruit fly *Drosophila melanogaster* [2,8], in the cockroaches *Diploptera punctata* [9] and *Blattella germanica* [10] and in the lepidopterans *Pseudaletia unipuncta* [11] and *Manduca sexta* [12]. In all cases, the precursor contains a single copy of the peptide. Moreover, all so far investigated lepidopteran myosuppressins have the same amino acid sequence: pQDVVHSFLRFamide [11,13].

Using immunohistochemistry [14–16] and *in situ* hybridization [9,17,18], myosuppressins have been localized in the central and stomatogastric nervous systems as well as in midgut endocrine-like cells. Myosuppressins are, therefore, a typical example of brain-gut peptides [19], and they have been shown to induce a variety of effects, including stimulation of skeletal muscle and heart contraction [13,20,21], stimulation of enzyme secretion from midgut [22,23] and inhibition of hindgut [3,4,7] and oviduct muscle contractions [5,6]. Myosuppressins have also been described as inhibitors of food intake in the cockroach *B. germanica*, where the peptide leucomyosuppressin (LMS) elicits antifeeding activity when injected in adult females at doses between 5 and 50 μg [3]. In addition, the distribution of ingested food along the different gut regions in LMS-treated specimens suggested a relationship between myoinhibitory properties and antifeedant activity [3].

Based on the observations on food intake reported in the omnivorous cockroach *B. germanica*, we wondered about possible antifeedant properties of myosuppressin in phytophagous insects,

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taking the Egyptian cotton leafworm, *Spodoptera littoralis*, as model. This generalist phytophagous species shows a broad geographical distribution, including Africa, Middle East and the Mediterranean area, and is considered a very important phytophagous agricultural pest, having cotton and different horticultural crops as main targets [24].

Here, we report the isolation of a cDNA coding for the precursor of *S. littoralis* myosuppressin. Molecular data have allowed gene expression studies in brain and midgut of this moth. Moreover, we have developed an immunoassay to quantify myosuppressin levels in tissues. Given that the results suggested a possible role for myosuppressin on feeding regulation in *S. littoralis*, antifeedant assays with the synthetic peptide were carried out. These assays revealed that myosuppressin has powerful antifeeding properties in this moth.

2. Materials and methods

2.1. Insect rearing

The Egyptian cotton leafworm *S. littoralis* (Boisduval) (Lepidoptera, Noctuidae) was reared on synthetic diet [25]. After egg hatching, larvae were placed in groups of 40–50 into 20 cm × 30 cm plastic boxes and maintained in a climatic chamber on a 16 h light: 8 h dark regime, at 25 ± 1 °C and 60–70% relative humidity. Freshly ecdysed penultimate (L5) and last (L6) instar larvae were selected every 24 h for further experiments. Under our rearing conditions, L5 lasts 3 days and L6, 4 days. At the end of L6 instar, larvae become prepupae, which are characterized by a dramatic body shortening. After two days (PP1, PP2), prepupae molt into pupae, and pupae were separated as white pupa (P0) and as P1, P2, P3…/… P11, at intervals of 24 h until day 11, where pupae transform into adults. Adults were fed with 20% sugar solution in water until eggs were laid out.

2.2. Cloning of S. littoralis myosuppressin cDNA

The cDNA of *S. littoralis* myosuppressin was obtained by PCR using a cDNA template extracted from brains of sixth instar larvae. An initial PCR was performed using a degenerate primer set (Lepmio-F1: TCCTCGAACTGTATGCTGAG, Lepmio-R1: AGGAACGAGTGC/TACA/GACG) designed on the basis of conserved sequences from lepidopteran myosuppressin precursors [11,13]. This yielded a fragment of approximately 120 bp, which was cloned and sequenced. Based on this sequence, specific primers were designed for 5′ and 3′RACE (5′-and 3′-RACE System Version 2.0; Invitrogen). To make sure that all amplifications raised with RACE correspond to the same molecule, the cDNA obtained was amplified with a forward primer designed at the 5′ end of the sequence and a reverse corresponding to the 3′ end just before the Poly $(A)^+$ tail. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced.

2.3. mRNA expression studies

Total RNA from brain and midgut samples was isolated using the GenElute Mammalian Total RNA Kit (Sigma). Aliquots of total RNA (0.5 μg for brain and 1 μg for midgut) were retrotranscribed as previously described [26]. Negative controls without the reverse transcriptase step were used to assess for possible genomic contamination. cDNA samples were subjected to PCR amplification with a number of cycles within the linear range of amplification, being in all cases between 30 and 35 cycles. For brain samples, the primer pairs, forward Lepmio-F1: TCCTCGAACTGTATGCTGAG, and reverse Lepmiobac-R1: TTGCGGTGGTTTGGCGTGGAC, were used to amplify a 189 bp fragment of the *S. littoralis* myosuppressin cDNA. For midgut samples, and due to background problems, we used a different forward primer, Lepmio-F5: TTCTGCCAGGCACTGAACAC. In this case, the sequence obtained was 215 bp long. As a reference, a fragment of cDNA corresponding to β-actin RNA of *S. littoralis* (Accession number: Z46873) using as forward: 5′-GTGATGGTTGGTATGGGTCAGAA-3′, and as reverse: 5′-GATCTGGGTCATCTTCTCCCTGT-3′, was amplified in parallel for each cDNA sample. cDNA probes for Southern Blot analyses were generated by PCR with the same primer pairs, using plasmid DNAs containing the corresponding cDNA clones as templates. The probes were labelled with fluorescein, using the Gene Images random prime-labelling module (Amersham Biosciences).

2.4. Preparation of conjugates

To raise an antibody against *S. littoralis* myosuppressin, the peptide pQDVVHSFLRFamide was conjugated to keyhole-limpet haemocyanin (KLH) (Sigma) at its C-terminus region following previously described methods [27], exposing the specific N-terminal region (antibody M-49). *S. littoralis* myosuppressin was also conjugated to BSA (Sigma) to coat the enzyme-linked immunosorbent assay (ELISA) plates. This ELISA uses an adsorbed myosuppressin-BSA conjugate that competes with sample peptide for binding to myosuppressin antibody. Once prepared, conjugates were dialyzed, lyophilized and stored at −20 °C.

2.5. Myosuppressin antibody

Three white male New Zealand rabbits were used to raise an antibody against *S. littoralis* myosuppressin-KLH as immunogen. Procedures were as described in [28]. Rabbits were injected subcutaneously on days 0 and 7 with 100 μg of immunogen diluted in 500 μl of Millipore water emulsified with 500 μl of Freud's complete adjuvant (Sigma). Subsequently, rabbits were injected on day 14 with the same dose of immunogen, but using Freud's incomplete adjuvant. Blood samples were obtained on day 21. Rabbits were boosted again once a month during 6 months. To assess the specificity of the *S. littoralis* myosuppressin antibody obtained (antibody M-49), we tested the cross-reactivity of it with *S. littoralis* myosuppressin (pQDVVHSFLRFamide), schistoFLRFamide (PDVDHVFLRFamide), drosomyosuppressin, (TDVDHVFLRFamide), leucomyosuppressin (pQDVDHVFLRFamide) and FMRFamide. For that purpose, we prepared standard curves of these peptides in PBST, and determined the corresponding EC_{50} values (concentration at which 50% of the antibody binding is inhibited). Antibody titer was determined by

measuring the binding of serial dilutions of the anti-*S. littoralis* myosuppressin serum to microtiter plates coated with 1 μg/ml of *S. littoralis* myosuppressin conjugated to BSA. To determine the optimal concentration of coating antigen and corresponding antisera, the two-dimension titration protocol described in [29] was carried out. Thus, binding of serial dilutions of M-49 antibody (1:500 to 1: 16,000) to microtiter plates coated with decreasing concentrations (6 to 0.09 μg/ml) of *S. littoralis* myosuppressin-BSA conjugate was measured.

2.6. Competitive ELISA

Polystyrene microtiter plates (96 wells) (Nunc Maxisorp, Roskilde, Denmark) were coated with *S. littoralis* myosuppressin-BSA conjugate (0.01 μg/ml) in coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) in a volume of 100 μl/well, and incubated overnight at 4 °C. Then, the plates were washed five times with PBST buffer and blocked with PVP 1% (Polyvinylpyrrolidone, Sigma) in PBST buffer. After 1 h, wells were washed again. The immunological reaction was then achieved by adding dilutions of the samples or standard peptide analyte in PBST buffer (from 10^{-4} M to10^{-11} M) in a volume of 50 μl/well. Subsequently, 50 μl of antibody diluted at 1/30,000 in PBST buffer were added to each well. After incubation at room temperature for 2 h with gentle shaking, plates were washed and goat antirabbit IgG peroxidase conjugated (Sigma) was added (diluted at 1/6000 in PBST, 100 μl/well). Plates were incubated for 1 h, washed again and 100 μl of substrate solution (12.5 ml of citrate buffer, 200 μl of 0.6% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide and 50 μl of 1% H_2O_2) was added to each well. Finally, plates were incubated for 8 min in the dark with gentle shaking. Colour reaction was stopped by adding 50 μl/well of 2 N H_2SO_4. Absorbance was read at 450 nm with a Titertek Multiscan Plus MKII spectrophotometer (Labsystems, Helsinki, Finland).

2.7. Sample preparation for ELISA

Brains and midguts were dissected under Ringer solution. Individual midguts were dissected free of Malpighian tubules, cut longitudinally and cleaned of its content. Haemolymph samples were obtained by cutting off one leg. Haemolymph was collected with a graduated micropipette and diluted in extraction buffer (methanol/acetic acid/water: 87:5:8). Then, the three tissues were processed with the same method. Each sample was homogenized in 200 μl of extraction buffer, the homogenate was centrifuged at 13,000 rpm for 15 min, and the pellet was re-extracted in 200 μl of the same buffer. Combined supernatants were evaporated, lyophilized and stored at −20 °C.

2.8. HPLC separation of haemolymph myosuppressin

For HPLC studies of circulating myosuppressin, haemolymph from 0-, 2- and 4-day-old *S. littoralis* sixth instar larvae, as well as from prepupae, was used. A volume of 1 ml of haemolymph was pooled from each age group (between 12 and 15 specimens were needed), extracted and lyophilized. Samples were diluted in water with 0.1% TFA and processed in a Merck Hitachi low-pressure system with automatic gradient controller (L-6200A) and UV–VIS detector (L-4200). The column was a C_{18} LiChrospher (Merck, Darmstadt, Germany) (4 mm × 125 mm, 5 mm particle size), supplied with a guard column using the same material. Separation was carried out under a gradient of H_2O/CH_3CN, both solvents having 0.1% TFA, and at a flow rate of 1 ml/min. The gradient used was 0–10% of CH_3CN in 5 min and then to 70% in 60 min. Synthetic *S. littoralis* myosuppressin was used as standard. Two-minute fractions were collected and lyophilized. Prior to be assayed by ELISA, fractions were resuspended in PBST buffer. In the above conditions, the synthetic peptide eluted at 24.1% of CH_3CN (at 31 min). To avoid contamination problems, we systematically ran a gradient without sample after each separation process.

2.9. Food consumption

Food consumption was measured according to previously described methods [30]. Isolated specimens of chosen ages were provided with a previously weighted portion of synthetic diet (fresh weight of initial food: FW). After 12 h, a new diet portion was provided, whereas the remains of the old portion were transferred to an oven at 60 °C, left there for 24 h and weighted (dry weight of final food: DW). In parallel, a weighted piece of food was placed in a control box, without any larvae, being processed as the other samples. Water lost to evaporation (evaporation factor: EF) estimated from these control experiments as DW/FW, was used as a correction factor. With these parameters, the dry weight of food consumed (food consumption: FC) was calculated from the formula: $FC = (FW \times EF) - DW$.

2.10. Antifeeding assays

Freshly ecdysed fifth instar larvae of *S. littoralis* were used in the antifeeding tests. Synthetic *S. littoralis* myosuppressin was injected into the haemocoel via the last proleg. The peptide was administered in 10% ethanol-Ringer's saline at doses of 50, 10, 1 or 0.1 μg, in a volume of 1 μl, using a Hamilton syringe. Controls received 1 μl of solvent. Antifeeding effects were studied following a non-choice test using leaf (*Lactuca sativa*) discs [31]. Freshly ecdysed fifth instar larvae that had been starved for 2 h were injected with the peptide and placed in a 55 mm diameter Petri dish containing 2 leaf discs of 1 cm^2 each. Then, the consumed area of the discs was quantified with an image analyzer at 1, 2, 3, 4, 5, 6 and 24 h.

3. Results

3.1. The cDNA of S. littoralis myosuppressin precursor

Using an RT/PCR approach followed by 5′ and 3′ RACE, we isolated a cDNA fragment encoding a myosuppressin precursor (GenBank accession number: AM778834). The conceptual translation of the peptide region gave a sequence compatible with the myosuppressin isolated in the lepidopterans *M. sexta* and *P. unipuncta* (pQDVVHSFLRFamide). Thus, the peptide region of *S. littoralis* precursor (Fig. 1A), begins with a glutamine

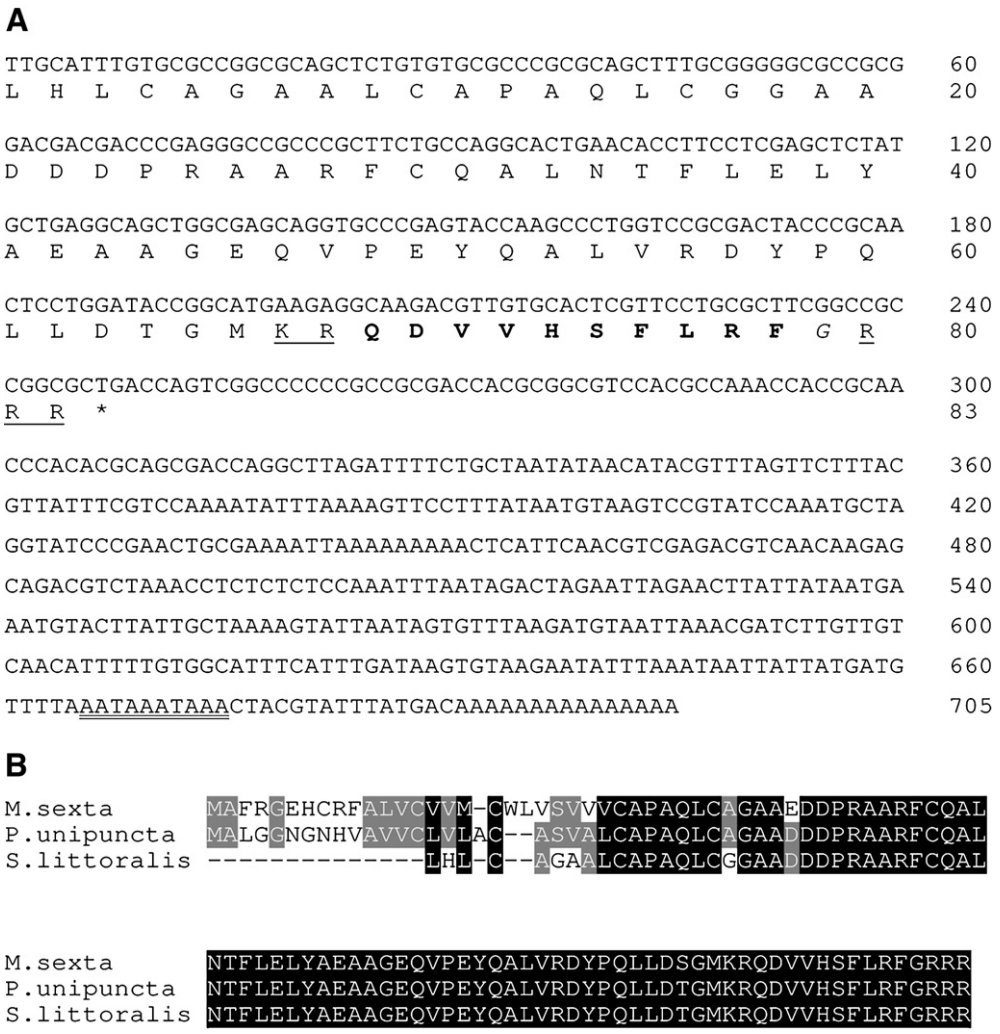

Fig. 1. Nucleotide sequence of *Spodoptera littoralis* myosuppressin cDNA, and deduced amino acid sequence of the protein precursor (A). The amino acid sequence corresponding to the peptide is shown in bold. Potential cleavage sites are underlined and the glycine necessary for amidation is shown in italics. Potential polyadenilation signal near the 3′ end of the sequence is double underlined. GenBank accession number: AM778834. Alignment (B) of the amino acid sequence of lepidopteran myosuppressin precursors of *Manduca sexta*, *Pseudaletia unipuncta* and *S. littoralis*. Grey boxes indicate residues having more than 50% identity, black boxes correspond to 100% identity.

residue (converted into pyroglutamate in the mature peptide), continues with the sequence DVVHSFLRF, and ends with a glycine residue (necessary for amidation). The myosuppressin sequence is flanked by the potential proteolytic cleavage sites KR and RRR, respectively.

As shown by the alignment (Fig. 1B), *S. littoralis* myosuppressin precursor is very similar to those of *P. unipuncta* (80.8% identity) and *M. sexta* (72.7% identity). However, the sequence of *S. littoralis* obtained in the present work is probably incomplete at the 5′end. The alignment suggests that about 16 residues are lacking. Towards 3′, a stop codon is located just after the RRR putative proteolytic cleavage site of the deduced peptide, and two overlapping polyadenylation sites are located 15 base pairs upstream from the poly $(A)^+$ tail.

3.2. *mRNA levels of* S. littoralis *myosuppressin*

Given that myosuppressins have been observed in the central and stomatogastric nervous system as well as in endocrine midgut cells, we investigated the levels of *S. littoralis* myosuppressin mRNA in brain and midgut during the last larval instar and prepupal stages of *S. littoralis* (Fig. 2), which are those where dramatic feeding fluctuations occur. In the brain, mRNA was detected at approximately constant levels in all life stages analyzed. In contrast, mRNA levels in the midgut were relatively low during days 0, 1 and 2 of the sixth instar larvae, increased from day 3 of the last instar larvae to the first day of prepupae, and vanished on the second day of prepupae (Fig. 2).

3.3. *ELISA measurements of* S. littoralis *myosuppressin*

To quantify *S. littoralis* myosuppressin levels, we developed a competitive indirect ELISA, using the antibody M-49 raised against the *S. littoralis* myosuppressin conjugated to KLH at its C-terminus. Antibody M-49 resulted to be very specific. It was reactive against *S. littoralis* myosuppressin, whereas it did not cross-react with the other related peptides (schistoFLRFamide, drosomyosuppressin, leucomyosuppressin and FMRFamide).

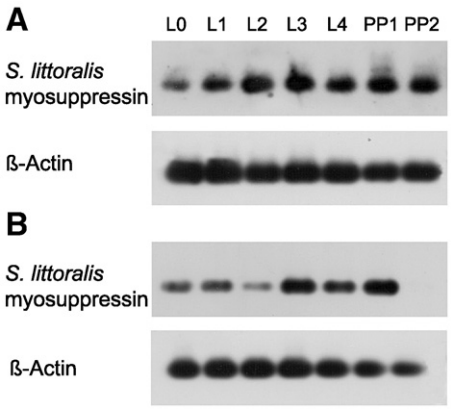

Fig. 2. Expression of *Spodoptera littoralis* myosuppressin mRNA in brain (A) and midgut (B) of *S. littoralis* staged on each day of the last larval instar (L0 to L4) and prepupae (PP1 and PP2). In all cases, *S. littoralis* β-actin levels were used as a reference. Southern blots shown are representative of three replicates.

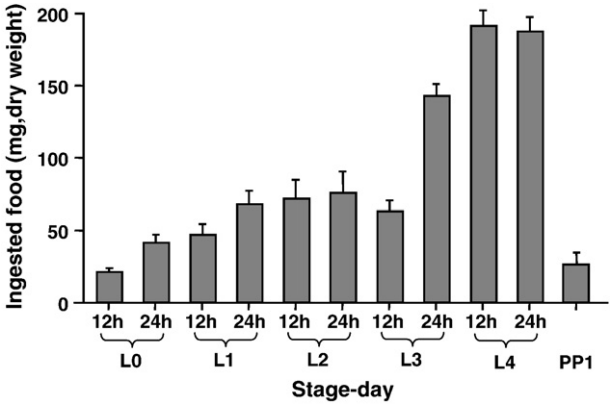

Fig. 4. Food intake pattern of *Spodoptera littoralis* quantified every 12 h on each day of the last larval instar (L0 to L4) and on day 1 of prepupal stage (PP1). Results are expressed as the mean±SEM (n=9–12).

Concerning the ELISA, the sensitivity was 242 ng and the detection limit 6.15 ng.

With this ELISA, we quantified *S. littoralis* myosuppressin levels in the brain, midgut and haemolymph of *S. littoralis* during the sixth larval instar and the prepupal stage. In the brain (Fig. 3A), *S. littoralis* myosuppressin levels were practically constant (between 3 and 4 ng/brain) from day 0 to day 2 of last larval instar, decreased dramatically on day 3, and increased again afterwards, practically recovering the initial larval values in the prepupal stage. In the midgut (Fig. 3B), levels were around 150 ng/midgut on days 0 and 1 of last larval instar, increased on day 2 (ca. 220 ng/midgut), and then decreased progressively until reaching ca. 40 ng/midgut in day 2 of prepupal stage. In the haemolymph, levels were around 6 ng/µl on day 0, decreased progressively until day 4 (ca. 2 ng/µl), and tended to increase in prepupal stage (Fig. 3C). In order to assess this pattern, and given the usually complex composition of haemolymph, we carried out a set of measurements where haemolymph samples were processed by HPLC prior to ELISA analyses. We used haemolymph pools of 1 ml from days 0, 2 and 4 of last instar larvae, and from the last day of prepupae. For all haemolymph samples analyzed, a single peak of *S. littoralis* myosuppressin immunoreactive material, coeluting with the synthetic peptide, was detected under the HPLC conditions described in Material and methods. Results indicate that *S. littoralis* myosuppressin levels in the haemolymph are high on freshly ecdysed last instar larvae (L0: 57.60 pg/µl), decrease progressively during the instar (L2=29.48 pg/µl; L4=11.95 pg/µl) and recover higher levels in the prepupae (PP2=25.80 pg/µl).

3.4. Food consumption in last larval instar and prepupae

In order to correlate *S. littoralis* myosuppressin patterns and feeding activity, we then measured food intake during the last larval instar and prepupal stage of *S. littoralis*. As in the characterization of myosuppressin patterns, we focused our study on the last instar larvae and prepupae, given that these are the life stages when growth fluctuates dramatically, presumably due to fluctuating feeding rates.

Food consumption was quantified at 12 h intervals. Results (Fig. 4) showed that food consumption was relatively low in freshly ecdysed last instar larvae and modestly increased until the first 12 h of day 3. However, on the last 12 h of day 3, food consumption increased dramatically, maintained very high levels on day 4, and suddenly decreased to very low values on the first day of prepupal stage. Food consumption in 2-day-old prepupae was too low to be measured by the method used.

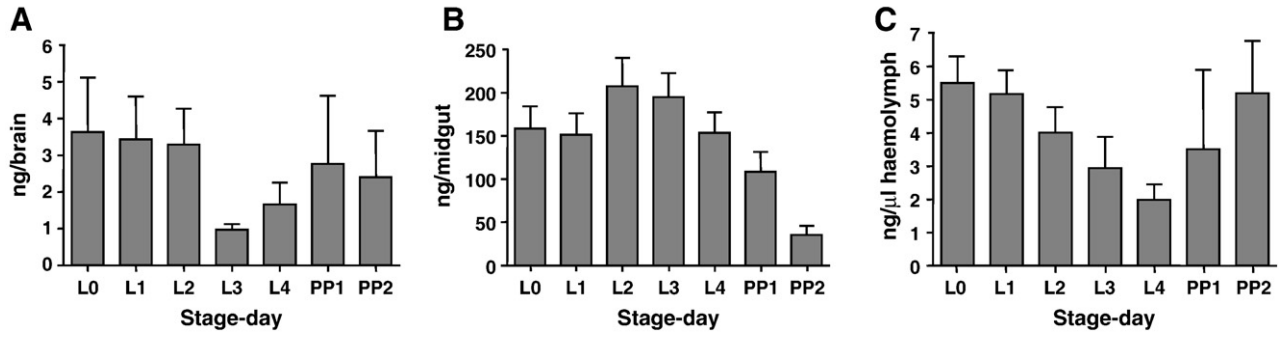

Fig. 3. Myosuppressin levels in crude extracts of brain (A), midgut (B) and haemolymph (C) of *Spodoptera littoralis*, on different days of the last larval instar (L0 to L4) and prepupae (PP1 and PP2). Levels were directly measured by ELISA and results are expressed as ng per brain or per midgut, or as ng per µl of haemolymph, and as the mean±SEM (n=6–11).

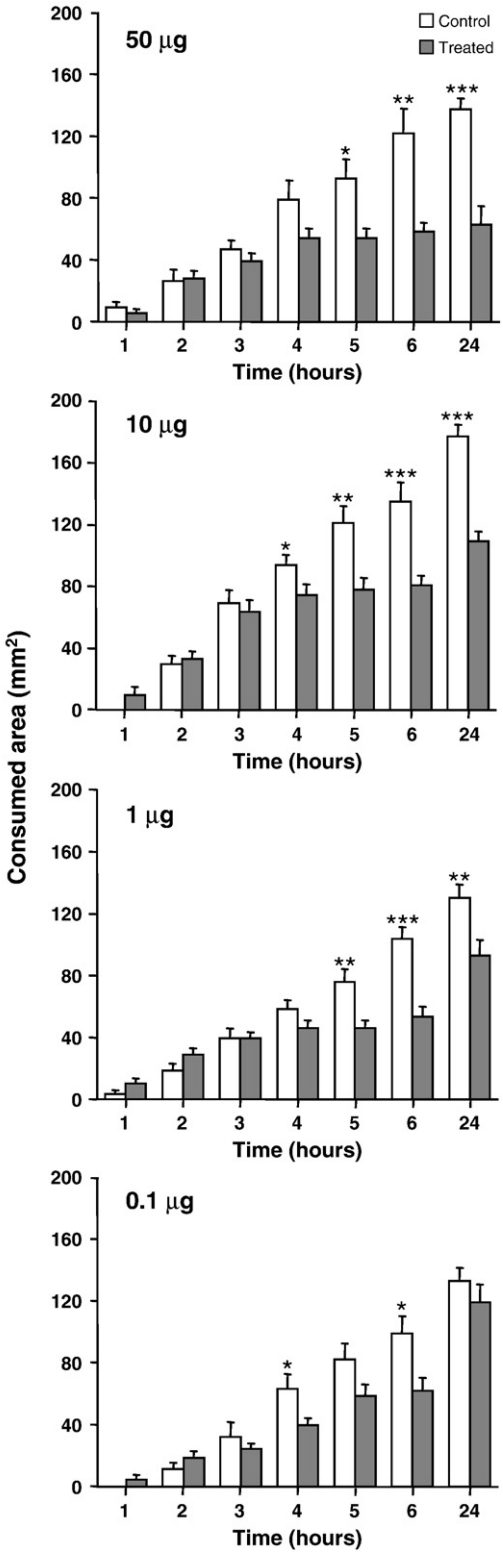

Fig. 5. Antifeeding bioassays carried out using non-choice leaf disks assays. *Spodoptera littoralis* myosuppressin was tested by injecting *S. littoralis* fifth instar larvae with synthetic peptide doses of 50, 10, 1 and 0.1 µg. Consumed areas were measured each hour during the first 6 h and 24 h after the injection. Results (mean±SEM, $n=10$) are expressed as consumed area in mm^2. The asterisks indicate significant differences with the respective control (*t*-test, $*P<0.05$, $**P<0.001$, $***P<0.0005$).

3.5. Antifeedant activity of S. littoralis myosuppressin

Comparison of *S. littoralis* myosuppressin levels (Figs. 2 and 3) with food consumption rates (Fig. 4) suggested a possible involvement of this peptide on feeding regulation. To address this hypothesis, we studied the effects of synthetic *S. littoralis* myosuppressin on feeding, using a non-choice leaf disc test. *S. littoralis* myosuppressin was tested at four doses: 50, 10, 1 and 0.1 µg, and leaf consumption was measured every hour during the first 6 h after the treatment, and at 24 h. Results (Fig. 5) showed that during the first 3 h, values of leaf consumption measured in treated larvae were undistinguishable from those of controls. Conversely, from 3 to 6 h, control larvae fed steadily whereas treated larvae practically stopped feeding at any of the concentrations tested. At 6 h, differences in leaf consumption between treated and controls were statistically significant in all experiments, and food intake inhibition was ca. 50%, irrespective of the dose used. At 24 h, the specimens treated with a dose of 50 µg, kept practically starved, and those treated with 10 µg still maintained severe food intake inhibition.

4. Discussion

Previous studies carried out in our laboratory had shown that leucomyosuppressin inhibits gut contractions as well as food intake in the omnivorous cockroach *B. germanica* [3], and we wondered whether this activity could be extended to phytophagous insects. We took the generalist phytophagous *S. littoralis* as model to study the possible role of myosuppressins as feeding regulators, given that lepidopteran myosuppressin effectively inhibits gut motility in this leafworm [10].

The first step was the characterization of myosuppressin in *S. littoralis*. Using a cDNA cloning approach, we identified the myosuppressin precursor of *S. littoralis* including the sequence of the peptide, which resulted to be identical to the other two myosuppressins described in lepidopteran species, that of *M. sexta* [12] and that of *P. unipuncta* [11]: pQDVVHSFLRFamide.

Myosuppressins are typical brain-gut peptides [19], as thoroughly demonstrated in *M. sexta*, where immunohistochemistry and *in situ* hybridization analysis localized ManducaFLRFamide mRNA in larval brain, nerve cord, neurohemal organs, midgut and in the enteric nervous system [12,32,33]. Thus, the expression pattern of *S. littoralis* myosuppressin was investigated in brain and midgut tissues, during the last larval instar, prepupae and pupae stages of *S. littoralis*. Results revealed that mRNA levels in the brain practically do not fluctuate, which is in agreement with equivalent data reported in *M. sexta* [12]. Conversely, *S. littoralis* myosuppressin mRNA levels in midgut form a broad peak covering the last days of last instar larvae and the first day of prepupae. The virtual absence of expression in late prepupae coincides with the onset of a massive cell death process in this organ, that culminates in the pupal stage [34].

To measure peptide levels, we raised an antibody against the N-terminal region of *S. littoralis* myosuppressin and we developed an ELISA. The antibody resulted to be extremely specific for this peptide, given that it did not cross-react with

other FMRFamide-related peptides. Until now, two myosuppressin ELISA have been reported [33,35] and both of them used as a primary antibody an anti-FMRFamide serum [36] which primarily recognizes FMRF/FLRF peptides.

mRNA and peptide patterns are practically parallel in midgut, and roughly symmetrical in the case of the brain, which suggests that the mechanisms regulating the expression, in terms of transcription and translation, are different in these two tissues. Moreover, *S. littoralis* myosuppressin levels in the haemolymph, either measured in crude as in prepurified extracts, exhibit a pattern similar to that of the brain. Lower levels measured in prepurified extracts result from much lower yield and perhaps from the occurrence of unspecific immunogens in crude extracts. In any event, the pattern is the same in both cases. Given that myosuppressin expression has been localized in the brain as well as in neurohemal organs in different insects [37], the parallel peptide patterns in brain and haemolymph, suggests that haemolymph *S. littoralis* myosuppressin derives mainly from brain sources.

The feeding pattern in last instar larvae and prepupae of *S. littoralis* indicates that there is an acute peak of food consumption on the penultimate and especially the last day of last instar larvae. Interestingly, the highest food consumption corresponds to the lowest levels of myosuppressin in brain and haemolymph. These correlations, led us to test the effects of *S. littoralis* myosuppressin on food consumption, and results showed that the synthetic peptide elicits a significant anti-feeding activity.

Taken together, the present results point to the hypothesis that myosuppressin regulates feeding in *S. littoralis*. If we additionally consider that *S. littoralis* myosuppressin inhibits gut motility in this species [10], we can propose that brain myosuppressin released through neurohemal organs to the haemolymph inhibits gut motility, and hence inhibits food intake, as occurs in the cockroach *B. germanica*. Therefore, the dynamics of circulating myosuppressin would modulate food intake, at least in the last larval instar of *S. littoralis*, where, as we have seen, highest levels of food intake coincide with lowest levels of brain and circulating myosuppressin.

This hypothesis does not exclude other possible roles of myosuppressin in *S. littoralis*, additional to those involving muscle contraction and feeding. For example, brain myosuppressin might be additionally involved in regulating ecdysteroid synthesis, as reported in the silkmoth, *Bombyx mori* [38,39], and gut myosuppressin might stimulate midgut amylase activity, as reported in other species [22,23].

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