Isolation and characterization of the B-type allatostatin gene of *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae)

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Junling Wang

P.R. China

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1. Gutachter: Prof. Dr. K.H. Hoffmann

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Prof. Dr. K. Dettner

Prof. Dr. B. Westermann

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To my parents, my wife and my daughter

Contents

Abbreviations

Amp	ampicillin
AMV	Avian myeloblastosis virus
AS	allatostatin
AT	allatotropin
bp	base pair
BCIP	5-bromo-4-chloro-3-indolylphosphat
BSA	Bovine serum albumin
CA	corpora allata
CaMV	Cauliflower mosaic virus
CC	corpora cardiaca
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
CSPD	Disodium 3-(4-metho xyspiro {1,2-dioxetane-3,2-(5-chloro) tricyclo [3.3.1.1^{3},7]decan}-4-yl)phenyl phosphate
dATP	2´-deoxyadenosine 5´- triphosphate
dCTP	2´-deoxycytidine 5´-triphosphate
dGTP	2´-deoxyguanosine 5´-triphosphate
dTTP	2´-deoxythymidine 5´-triphosphate
dNTPs	mixture of dATP, dCTP, dGTP and dTTP
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	2´-deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EDTA	ethylenediamine tetraacetic acid
E. coli	*Escherichia coli*
Fig	figure
g	gram, or standard gravitation
HPLC	high performance liquid chromatography
IOD	integrated optical density

IPTG	isopropyl-β-D-thiogalactoside
JH	juvenile hormone
LB	Luria-Bertani-Medium
kDa	kilodalton
L	liter
M	molar concentration
min	minute
mRNA	messenger RNA
NBT	nitro blue tetrazolium chloride
NCA	nervus corporis allati
NCC	nervus corporis cardiaci
OD	optical density
PCR	polymerase chain reaction
PTSP	prothoracicostatic peptide
PTTH	prothoracicotropic hormones
RACE	Rapid Amplification of cDNA Ends
RNA	ribonucleic acid
RNAi	RNA-mediated interference
RNase	ribonuclease
rpm	rounds per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
sec	second
SOG	suboesophageal ganglia
ssDNA	single-stranded DNA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
Tris	tris-(hydroxymethyl)-aminomethane
U	unit
UV	ultra violet
V	volt
% (v/v)	volume percent per volume

% (w/v)	weight percent per volume
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1. Introduction

Juvenile hormones (JH) and ecdysteroids play a crucial role in the regulation of development and reproduction of insects. These hormones control moulting and metamorphosis in the laval stages, whereas in adult insects, they regulate vitellogenesis and ovarian development in females, and spermatogenesis and growth of the accessory reproductive glands in males (Koeppe et al., 1985; Nijhout,1994; Riddiford,1994; Gäde et al., 1997).

Juvenile hormone III (JH III), the only JH which has been identified in crickets (*Gryllus bimaculatus*) so far (Koch and Hoffmann, 1985), is synthesized and released from the corpora allata (CA) which are present in all insect developmental stages. Ecdysteroids are synthesized in the prothoracic glands of larval and pupal stages, but these glands generally degenerate during or shortly after adult moulting. In adult female crickets, ecdysteroids are synthesized in the ovaries and in the abdominal integument plus adjacent segmental fat body (Delbecque et al., 1990; Hoffmann et al., 1992; Weidner and Hoffmann, 1992; Weidner et al., 1992).

It is reported that the ecdysteroid titres in the haemolymph of female crickets which had been kept under suboptimal rearing conditions were substantially lower than in females reared under optimal conditions (Hoffmann et al., 1981). Moreover, suboptimal rearing temperatures led to a reduced rate of ecdysteroid biosynthesis (Weidner et al., 1992). As with the ecdysteroid biosynthesis, JH biosynthesis was reduced under suboptimal rearing temperatures (Espig and Hoffmann, 1985). Biosynthesis of ecdysteroids and JH may affect each other: removal of the CA (allatectomy) and the following reduction of JH titres in the haemolymph of the animals led to reduced rates of ecdysteroid biosynthesis (Hoffmann and Gerstenlauer, 1997). Removal of the ovaries resulted in lower ecdysteroid titres in the haemolymph and in turn inhibited JH biosynthesis (Wennauer et al., 1989). In addition, other results indicated a positive feedback between JH titer and JH biosynthesis and ecdysteroid titres and ecdysteroid biosynthesis, respectively, in adult crickets (Wennauer and Hoffmann, 1988; Wennauer et al., 1989; Hoffmann and Gerstenlauer, 1997).

The question is, which factors regulate the production of the two insect (cricket) glandular hormones?

1.1. Ecdysio- and allatoregulating factors

Since around 1980, interest has focused on the isolation, purification and identification of factors that regulate the biosynthesis and release of the insect glandular hormones (for review see Gäde et al., 1997; Hoffmann, 2003). The research work mainly based on the assumption that such compounds may be helpful in designing safer and more specific insecticides (Couillaud and Peypelut, 1995; Hoffmann and Lorenz, 1998). Ecdysteroid biosynthesis in the prothoracic glands is stimulated by peptide hormones called ecdysiotropins or prothoracicotropic hormones (PTTH). The structure and mode of action of such hormones is known from a few species, mainly silkmoths. PTTH occurs in multiple forms-- large (ca. 22 kDa) and small PTTHs (ca. 4 kDa). Only two peptides with ecdysiostatic activity have been described so far (Bylemans et al., 1994; Hua et al., 1999).

On the basis of their ability to stimulate or inhibit JH biosynthesis *in vitro* by the CA, several neuropeptides were classified as allatotropins (stimulatory) or allatostatins (inhibitory). Allatotropins and allatostatins may reach the glands either via haemolymph or via nervous connections (Goodman, 1990; Stay et al., 1994a).

1.2. Allatotropins

To date, only one allatotropin (AT) is known, the *Manduca sexta* (Manse-AT, GFKNVEMMTARGF-NH_2) allatotropin (Kataoka et al., 1989). This peptide was isolated from the head of pharate adults of the moth *M. sexta* and stimulated the JH biosynthesis in adult animals but did not affect CA activity of larvae or pupae. Synthetic allatotropin did not activate the CA of the beetle *Tenebrio molitor*, the locust *Schistocerca gregaria*, or the cockroach *Periplaneta americana*, but stimulated the CA of the noctuid moths *Heliothis virescens* (Kataoka et al., 1989), *Lacanobia oleracea* (Audsley et al., 1999), and *Spodoptera frugiperda* (Oeh et at., 2000), suggesting some order specificity. In addition to its stimulatory effect on JH biosynthesis, Manse-AT is a potent cardioaccelerating peptide in pharate adults of *M. sexta* (Veenstra et al., 1994). Studies with NH_2-terminal truncated sequences of the synthetic peptide suggested that the amino acids 6-13 represent the biologically active core. An allatotropin immunoreactive peptide was isolated from the mosquito *Aedes aegypti* and its structure determined as APFRNSEMMTARGF-NH_2. Its cDNA sequence was identified by Veenstra and Costes (1999).

In the cricket *G. bimaculatus*, methanolic extracts of suboesophageal ganglia (SOG) from adult males and females exerted allatotropic activity depending on the sex and the age of the donor animals. A maximal allatotropic effect was observed when testing SOG-extracts from 3 day old females, an age at which JH biosynthesis is also maximal. SOG-extracts from males showed a less pronounced age-dependent allatotropic effect, and the maximal stimulation obtained with these extracts was also lower than with SOG-extracts from females (Lorenz and Hoffmann, 1995). SOG-extracts from *G. bimaculatus* stimulated the JH biosynthesis of CA from house crickets, *Acheta domesticus*, and *vice versa*, suggesting that the allatotropic effect is not species-specific. The allatotropic factor proved be be heat-stable and proteinase-sensitive, suggesting its peptide nature. Unfortunately, the allatotropic factor from the cricket SOG could not be completely purified and sequenced.

Manse-AT may play different roles in larval and adult insects (Lee et al., 1998). The peptide rapidly inhibits active ion transport *in vitro* across the midgut epithelium of 2 day old (feeding) fifth instar tabacco hornworms, and this inhibition was reversible. Midguts from pharate fifth instars or wandering fifth instars were not affected by the peptide.

The genes encoding Manse-AT were cloned from *M. sexta* (Taylor et al., 1996), *Pseudaletia unipuncta* (Truesdell et al., 2000), *Bombyx mori* (Park et at., 2002) and *S. frugiperda* (Abdel-latief et al., 2003). In *M. sexta* and *S. frugiperda*, the gene is expressed in at least three mRNA isoforms in a tissue-specific manner that differ from each other by alternative splicing (Horodyski et al., 2001; Abdel-latief et al., 2004).

Immunostaining studies with a Manse-AT antibody indicated that the peptide was present in the retrocerebral complex, the brain, and the ventral nerve cord of *M. sexta* (Veenstra and Hagedorn, 1993). In larvae of *M. sexta*, with the techniques of whole-mount *in situ* hybridization and whole mount immunohistochemistry, abundant Manse-AT mRNA and immunoreactivity were detected in cells of the frontal ganglion and the terminal abdominal ganglion, whereas in the brain and SOG, lower levels of Manse-AT mRNA were found. In the pupal and pharate adult stages of *M. sexta*, Manse-AT mRNA and immunoreactivity were detected in cells of the abdominal ganglia and in additional cells of the terminal abdominal ganglion (Bhatt and Horodyski, 1999). The expression pattern confirms that Manse-AT

exhibits multiple (pleiotropic) functions during the life cycle of the insect, some of which may be specific to a particular life stage.

1.3. Allatostatins

The name allatostatin was originally introduced to define unknown regulatory factors that inhibited JH biosynthesis *in vitro* by the CA of insects (Tobe, 1980). When these factors were first isolated from brain extracts of the cockroach *Diploptera punctata* (Woodhead et al., 1989; Pratt et al., 1989) and identified as neuropeptides, the term allatostatin was retained. So far, more than 60 allatostatins have been isolated and characterized from a variety of insect species. These peptides can be classified into three groups: the lepidopteran (*M. sexta*) allatostatin (C type), the FGL- allatostatin superfamily (A type), and the $W(X)_6W$ allatostatins (B type).

1.3.1. The lepidopteran (*M. sexta*) allatostatin (C type)

A non-amidated allatostatin of pEVRFRQCYFNPISCF-OH (Manse-AS), which was purified from the brains of *M. sexta*, strongly inhibited JH biosynthesis *in vitro* by the CA of fifth instar larvae and adult females of the moth (Kramer et al., 1991). It also had an inhibitory effect on the activity of the CA from adult females of the lepidopterans *Heliothis virescens, H. zea*, and *L. oleracea* (Kramer et al., 1991; Audsley et al., 1999, 2000; Edwards et al., 2001; Teal, 2002), but did not inhibit the CA from adult females of the beetle *T. molitor*, the grasshopper *Melanoplus sanguinipes*, or the cockroach *P. americana* (Kramer et al., 1991). Such as Manse-AT, Manse-AS seems to exhibit an allatoregulatory effect only in lepidopterans (Weaver et al., 1998).

Synthetic Manse-AS did not affect the rate of JH secretion *in vitro* from the CA of adult *S. frugiperda*, but it reduced the JH biosynthesis of CA which had previously been activated by Manse-AT. This allatostatic effect of Manse-AS on allatotropin-activated glands was dose-dependent and reversible (Oeh et al., 2000).

The genes encoding Manse-AS were cloned from *P. unipuncta* (Jansons et al., 1996), *Drosophila melanogaster* (Williamson et al., 2001b), and *S. frugiperda* (Abdel-latief et al., 2003). In *P. unipuncta,* the gene is present as a single copy per haploid genome. Expression

of this gene was low in sixth instar larvae, prepupae and early pupae but relatively high in late pupae, and day 1 and 3 adults of both sexes (Jansons et al., 1996). In *D. melanogaster*, strong gene expression was detected in larval and adult stage of flies, but less in pupae and embryos (Williamson et al., 2001b). In the fall armyworm *S. frugiperda*, gene expression was detected in the brains of larvae, pupae and adults, but also in tissues such as the midgut and the ovaries (Abdel-latief et al., 2004). In the tomato moth *L. oleracea*, Manse-AS-like immunoreactivity was distributed throughout the central nervous system and associated with midgut and Malpighian tubules (Audsley et al., 1998).

1.3.2. The FGL-allatostatin superfamily (A type)

Allatostatins of the FGLamide type were first isolated from brain extracts of the cockroach *D. punctata* (Woodhead et al., 1989). Thereafter, further members of this peptide family were isolated from other cockroaches of various families (Weaver et al., 1994; Bell9s et al., 1994), but also from the cricket *G. bimaculatus* (Lorenz et al., 1995b), the locust *S. gregaria* (Veelaert et al., 1996), the stick insect *Carausius morosus* (Lorenz et al., 1998a, 2000), the blowfly *Calliphora vomitoria* (Duve et al., 1993), the mosquito *A. aegypti* (Veenstra et al., 1997), and the lepidopteran species *Cydia pomonella, Helicoverpa armigera* (Duve et al., 1997a), and *M. sexta* (Davis et al., 1997). Recently, allatostatins were also reported in crustaceans (Duve et al. 1997b, 2002, Dircksen et al., 1999). Members of this peptide family are 5 to 18 amino acids long and contain the conserved C-terminus Y/FXFGL/I/V-amide, which is essential for their inhibitory effect on the JH biosynthesis (Hayes et al., 1994). An unusual variation of the allatostatin sequence, GPPYDFGM-amide, was found in the blowfly (Duve et al., 1993).

The inhibiting action of the FGL-allatostatins on JH-release *in vitro* by the CA is rapid and in a fully manner, but seems to be restricted to cockroaches and crickets (Woodhead et al., 1989; Pratt et al., 1991; Bell9s et al., 1994; Weaver et al., 1994; Woodhead et al., 1994; Lorenz et al., 1995b). Effective concentrations causing a 50% inhibition ranged from 10^{-10} to 10^{-7} M. In crickets, the maximum inhibition (60-80%) of JH III release was reached with a concentration of about 10^{-7} M, 50% inhibition of JH release was obtained with 0.4 to $3x10^{-8}$ M. The FGL-allatostatins isolated from stick insect, locust, flies and moths also inhibited JH biosynthesis by the CA of cockroaches and crickets, but were not at all active when using CA of the donor species (reviewed in Hoffmann et al., 1999).

In addition to the inhibiting action on JH biosynthesis, FGL-allatostatins exhibited multiple functions. An inhibition of visceral muscle contraction was demonstrated for the hindgut (Lange et al., 1993) and foregut (Duve et al., 1995) of cockroaches, and the ileum of blowflies (Duve and Thorpe, 1994). An inhibition of the spontaneous oviduct contraction was found in the locust *S. gregaria* (Veelaert et al., 1996). In the cockroach *Blattella germanica*, FGL-allatostatins affected egg development by inhibiting the the vitellogenin production (Martin et al., 1996) and the release of vitellogenin from the periovarian fat body (Martin et al., 1998).

Immunohistochemical localization with a monoclonal antibody raised against *D. punctata* allatostatin 7 (Dippu-AS 7) indicated that allatostatin immunoreactive cells were localized in nerve fibers in the brain/retrocerebral complex of the cockraches *D. punctata* (Stay et al., 1992) and *P. americana* (Agricola et al., 1992), and in the crickets *G. bimaculatus* and *A. domesticus* (Neuhäuser et al., 1994; Witek et al., 1999). These results suggested that the allatostatins are produced in lateral neurosecretory cells of the brain and are delivered to the CA through the nervi corporis cardiaci (NCC) II. Strong immunoreactivity was detected in four large anterior medial cells of the pars intercerebralis, but no immunoreactivity was detected in the NCC I from the medium neurosecretory cells. Immunoreactivity localized in the corpora cardiaca (CC) suggested this neurohaemal organ as a site of release of allatostatins into the haemolymph. Immunoreactive cells were also detected in the suboesophageal and abdominal ganglia of cockroaches. In the cricket *G. bimaculatus*, FGL-allatostatin immunoreactivity was detected in the cortical cytoplasm of previtellogenic and vitellogenic oocytes. FGL-allatostatin immunoreactivity could also be detected in the nerves of the antennal pulsatile organ and in hindgut muscles of the cockroaches *D. punctata* and *P. americana* (Woodhead et al., 1992; Lange et al., 1993) as well as in endocrine cells of the midgut of *D. punctata* (Reichwald et al., 1994; Yu et al., 1995a). Moreover, FGL-allatostatin or allatostatin-like immunoreactivity was demonstrated in the brain/retrocerebral complex, nerves, ganglia, or endocrine cells of the midgut from locusts, blowflies, fly moths and an earwig species, and even in many non-arthropods (reviewed in Hoffmann et al., 1999).

The first FGL-allatostatin gene was identified from the cockroach *D. punctata* (Donly et al., 1993). Subsequently, another FGL-allatostatin gene was isolated from a distantly related cockroach *P. americana* by PCR, both from the genomic DNA and a brain-derived cDNA (Ding et al., 1995). The two pre-proallatostatins are similar in sequence, size and organization. The precursors are organized in several domains, beginning with a hydrophobic signal

peptide domain followed by the allatostatin peptides which are flanked by G-K/R-R endoproteolytic cleavage sites, and four acidic spacer regions which deparate the allatostatins into five clusters and probably serve as compensation to the basic charge contribution of the cleavage sites. Thereafter, FGL-allatostatin gene sequences were isolated from the cockroaches *B. germanica*, *Blatta orientalis*, *Supella longipalpa*, *Blaberus craniifer* (Bell9s et al., 1999), the locust *S. gregaria* (Vanden Broeck et al., 1996), the dipterans *C. vomitoria*, *Lucilia cuprina* (East et al., 1996), and *A. aegypti*, (Veenstra et al., 1997), and the lepidopteran species *H. armigera* (in Duve et al., 1997a), *D. melanogaster* (Lenz et al., 2000a) and *B. mori* (Secher et al., 2001). In the cockroach species, the FGL-allatostatin gene sequences contain 12 to 14 different allatostatins, and are similar in size and organization. However, the number of hormones encoded by the allatostatin precursors seems to be reduced during the evolution of insects. In the locust (Caelifera), the number of allatostatins is reduced to 10-12, and the precursors of the most derived order, the Diptera, include only 4-7 peptides. In all investigated species the allatostatins seem to be derived from a single polypeptide precursor (Bendena et al., 1997). Only the blowfly seems to have two FGL-allatostatin genes: one encoding the allatostatins with a terminal leucyl-amide and the other one encoding the methionyl-amide allatostatins (East et al., 1996).

The high number of homologous peptides within one prohormone as well as high variability both in number and sequence of the allatostatins as observed in species from different orders suggest that the FGL-allatostatin gene sequences were generated through a process of internal gene duplication which occurred before these species diverged from each other in evolutionary time (Bell9s et al., 1999), and that the processes of gene duplication and mutation may have conferred functional advantages that were selected during evolution (Duve et al., 1997b). Functions of the allatostatins seem to be more diversified in the primitive insects such as cockroaches and crickets, where peptide variability is also high, whereas they lost their allatostatic action in the more advanced insect orders (Lepidoptera, Diptera), where structural variability is also lower.

The gene encoding FGLamide allatostatins from the cricket *G. bimaculatus* has recently been isolated in our laboratory (Meyering-Vos et al., 2001). The hormone precursor is similar in size and arrangement of the peptides to those from cockroaches with a total number of 14 allatostatins. A single mutation/deletion in the coding region between the third and fourth acidic spacer seems to have reduced the number of peptides from 15 to 14. Different from the

cockroach precursors, the cricket prohormone contains one peptide (AGGRQYGFGL-NH_2) in two copies. By RT-PCR, gene expression was detected in various tissues of adult virgin and mated females, such as the brain, the suboesophageal ganglion, the caecum, ileum, midgut, and colon, the fat body, the ovary, and the female accessory reproductive glands in a tissue- and time-specific manner (Meyering-Vos and Hoffmann, 2003).

1.3.3. The $W(X)_6W$-allatostatins (B type)

Five nonapeptides which inhibited JH biosynthesis *in vitro* of the CA were isolated from the the brains of the cricket *G. bimaculatus* by conventional chromatographic techniques (Lorenz et al., 1995a, 1999; Hoffmann and Lorenz, 1997; Lorenz and Hoffmann, 1998). These C-terminally amidated nonapeptides contain the amino acid tryptophan at positions 2 and 9 and show high sequence similarity to the myoinhibiting peptides previously isolated from *L. migratoria* (Locmi-MIP) (Schoofs et al., 1991) and *M. sexta* (Blackburn et al., 1995). These peptides have been designated W^2W^9-allatostatins or B type allatostatins. Neuropetides with a similar structure were also isolated from the stick insect *C. morosus* (Lorenz et al., 2000) and the silkworm *B. mori* (Hua et al., 1999). Five B type allatostatins were deduced from a cDNA sequence of *D. melanogaster* (Williamson et al., 2001a). Three of them turned out to be deca- and dodecapeptides and one of them was N-terminally blocked by a pyroglutamate. However, the positions of the two Try-residues were conserved. Peptides of this family that contain more than nine amino acids were also found in the corresponding prohormone sequence of *B. mori* (Hua et al., unpublished data; sequence submitted to Genbank accession no. AB073553) (see Table. 1).

In *G. bimaculatus*, the inhibiting action of the B type allatostatins was rapid and reversible with an efficiency similar to that of the FGLamide allatostatins. Even after long exposure of the CA to a relatively high dose (10^{-6} M) of B type allatostatins, the glands recovered completely from inhibited rates after the peptides had been withdrawn from the incubation medium. However, the potency of the B type allatostatins was lower than that of the A type allatostatins. The B type allatostatins caused 50% inhibition of JH biosynthesis at doses that were up to one order of magnitude higher compared to the A type allatostatins. No synergistic effect of the allatostatins from the two peptide families was observed. An allatostatic activity of B type allatostatins was also detected in the house cricket *Acheta domesticus* (Lorenz et al., 1995a). The B type allatostatins isolated from the stick insect *C.*

morosus (Lorenz et al., 1998a, 2000) inhibited CA activity in crickets but had no effect on the CA of the stick insect itself. Thus the allatostatic function of the B type allatosatins seems to be restricted to crickets (Lorenz et al., 1997a).

Like other allatostatins, the $W(X)_6W$ allatostatins seem to act multifunctionally. Myoinhibiting functions were detected in *L. migratoria* (Lom-MIP) (Schoofs et al., 1991), *Leucophaea maderae* (Hoffmann et al., 1998), *P. americana* (Predel et al., 2001) and *M. sexta* (Blackburn et al., 1995). In addition to their allatostatic function in crickets, an ecdysiostatic function of this peptide in *G. bimaculatus* was shown. Two of the B type allatostatins could effectively inhibit the ovarian ecdysteroid biosynthesis in a dose-dependent manner (Lorenz et al., 1997b). A prothoracicostatic (ecdysiostatic) peptide which shares the conserved $W(X)_6W$ structure was isolated from larval brains of *B. mori* (Bommo-PTSP; Hua et al., 1999).

Immunohistochemical studies with *G. bimaculatus* B type allatostatin antibodies showed immunoreactive material in the CC as well as in the arborizing nerve fibers within the CA of adult female crickets (Witek et al., 1999). In *L. migratoria*, an antiserum raised against Lom-MIP was used to study the distribution of immunoreactivity in the nervous system (Schoofs et al., 1996). Immunoreactive fibers were detected in the NCC II, within the glandular part of the CC, and also in the NCA I connecting the CC to the CA and arborizing there. In *D. melanogaster*, gene expression was detected in all developmental stages, but weakly in embryos and strongly in larvae. *In situ* hybridization studies with larvae indicated that the gene is expressed in neurons of the brain, in abdominal ganglia and in endocrine cells of the gut (Williamson et al., 2001a).

Table. 1 Structures and actions of known $W(X)_6W$ allatostatins

	Amino acid sequence	Name	Demonstration	Functions
DICTYOPTERA				
Periplaneta americana	GWQDLQGGWamide	Peram-MIP	sequ.	Myoinhibiting
PHASMATODEA				
Carausius morosus	AWQDLQGGWamide	Carmo-AS B1	sequ.	Inhibiting JH biosynthesis in the CA of *Gryllus,* but not in the CA of *Carausius*
	AWQDLNTGWamide	Carmo-AS B2		
	GWQDLQSGWamide	Carmo-AS B3		
	AWQDLQGAWamide	Carmo-AS B4		
	AWQDLQAGWamide	Carmo-AS B5		
	AWQDLGSAWamide	Carmo-AS B6		
ENSIFERA				
Gryllus bimaculatus	GWQDLNGGWamide	Grybi-AS B1	sequ., immun.	Inhibiting JH biosynthesis in the CA; inhibiting ovarian ecdysteroid biosynthesis
	GWRDLNGGWamide	Grybi-AS B2		
	AWRDLSGGWamide	Grybi-AS B3		
	AWERFHGSWamide	Grybi-AS B4		
	AWDQLRPGWamide	Grybi-AS B5		
CAELIFERA				
Locusta migratoria	AWQDLNAGWamide	Locmi-MIP	sequ., immun.	Myoinhibiting
DIPTERA				
Drosophila melanogaster	AWQSLQSSWamide	drostatin-B1	cDNA	Unknown
	AWKSMNVAWamide	drostatin-B2		
	EAQGWNKFRGAWamide	drostatin-B3		
	EPTWNNLKGMWamide	drostatin-B4		
	DQWQKLHGGWamide	drostatin-B5		
LEPIDOPTERA				
Manduca sexta	AWQDLNSAWamide	Manse-MIP1	sequ.	Myoinhibiting
	GWQDLNSAWamide	Manse-MIP2		
	APEKWAAFHGSWamide	Manse-MIP3		
	GWNDMSSAWamide	Manse-MIP4		
	GWQDMSSAWamide	Manse-MIP5		
	AWSALHGAWamide	Manse-MIP6		
Bombyx mori				
	AWQDLNSAWamide	Bommo-PTSP1	sequ., cDNA*	Inhibiting ecdysteroid biosynthesis of prothoracic gland
	AWQDMSSAWamide	Bommo-PTSP2		
	GWQDLNSAWamide	Bommo-PTSP3		
	AWSSLHSGWamide	Bommo-PTSP4		
	AWSALHGTWamide	Bommo-PTSP5		
	GWNDISSVWamide	Bommo-PTSP6		
	SWQDLNSVWamide	Bommo-PTSP7		
	APEKWAAFHGSWamide	Bommo-PTSP8		

Abbreviations: sequ., peptide isolated and sequenced; immun., immunological demonstration; cDNA, cDNA isolated and sequenced; cDNA*, peptides deduced from the cDNA which only was submitted to Genbank and the corresponding names are designated temporarily by the author of this thesis for convenient description. Combined from Blackburn et al. (1995, 2001), Hoffmann et al. (1998), Hua et al. (1999) and sequence of

accession no. AB073553, Lorenz et al. (1995a, 1999, 2000), Predel et al. (2001), Schoofs et al. (1991,1996), Williamson et al. (2001a), Witek et al. (1999), Witek and Hoffmann (2001).

1.4. Allatostatin receptors

The presence of multiple allatostatins and the multifunctional character of allatostatins might suggest the existence of individual receptors for each substance. Allatostatin receptors have been identified and partially characterized from both brain/retrocerebral complexes (Yu et al., 1995b) and gut membrane preparations of *D. punctata* (Tobe et al., 1998), or deduced from gene sequences of *D. melanogaster* (Birgül et al., 1999; Lenz et al., 2000b, 2000c), *B. mori* (Secher et al., 2001), and *P. americana* (Auerswald et al., 2001). However, whether each allatostatin species is associated with a different receptor/receptor subtype remains to be determined.

The first allatostatin A type receptor gene was recently cloned in *D. melanogaster* independently by two groups (Birgül et al., 1999; Lenz et al., 2000b). The co-expression of this receptor with G-protein gated inwardly rectifying potassium channels (GIRK) in frog oocytes led to an assay system that was used to screen *D. melanogaster* brain extracts for an active ligand of this receptor and finally led to the isolation of this FGL-allatostatin receptor (Birgül et al., 1999). Lenz et al. (2000c) identified a second A type allatostatin receptor in *Drosophila* (DAR-2). Receptors similar to those from *Drosophila* were cloned from the cockroach *P. Americana* (Auerswald et al., 2001) and the moth *B. mori* (Secher et al., 2001). Comparing the allatostatin receptor gene of *B. mori* with the two receptor genes of *D. melanogaster* indicates that the two introns of the *B. mori* gene occur at exactly the same positions and have the same intron phasing as the two introns in the *D. melanogaster* receptor genes, suggesting that the three receptors are not only structurally, but also evolutionarily related (Secher et al., 2001). Structurally, the transmembrane regions of the receptors are most closely related to the mammalian galanin receptors. Southern blots suggested the existence of one or more additional A type allatostatin receptors also in *Bombyx* (Secher et al., 2001). Northern blots and quantitative RT-PCR indicated that the allatostatin receptor gene is mainly expressed in the gut and to a much lesser extent in the brain of *B. mori*.

Kreienkamp et al. (2002) identified two novel orphan G-protein coupled receptors from *D. melanogaster* which are related to the mammalian opiod/somatostatin receptor family and

were activated by a *Drosophila* C type allatostatin. No B type allatostatin receptors are known so far.

1.5. Biochemical and molecular action of allatostatins

Studies of biochemical and molecular action of allatostatins focused on their mechanisms of inhibition of JH biosynthesis. Two approaches can be taken to study the mode of action of allatostatins on the CA: analyzing the biochemical steps involved in the *de novo* synthesis of JH to determine which one(s) is/are affected by the incubation of the CA with the allatostatins (Sutherland and Feyereisen, 1996) or binding assays for peptides to putative receptors in order to elucidate the second messenger responses and the ultimate biochemical target(s).

JH biosynthesis starts with cytosolic acetyl-CoA as substrate (Schooley and Baker, 1985). In the CA of *D. punctata* this cytosolic acetyl-CoA pool is mainly fed by C_2-units that originate from the mitochondria and are transported into the cytosol by the citrate/malate shuttle (Sutherland and Feyereisen, 1996). FGLamide allatostatins seem to inhibit the transfer of the C_2-units, thus acting on a very early step of JH. In the cricket *G. bimaculatus*, members of both peptide families (FGL- and $W(X)_6W$-amides) inhibited the last step(s) of JH biosynthesis. For example, Grybi-AS A5 and B5 decreased the methyl farnesoate accumulation in the CA of adult females which had been incubated in the medium containing 200 µM farnesol (Lorenz et al., 1999).

Signal transduction within the CA occurs by way of known second messengers, including cyclic nucleotides, Ca^{2+}, and the phosphoinositoles (Rachinsky and Tobe, 1996). Differences in signal transduction between species may reflect differences in the peptide signals that regulate JH biosynthesis and in the intrinsic state of the CA. In general, the array of allatostatins and multiple receptor subtypes raise the possibility of multiple signal transduction mechanisms for these peptides.

1.6. The aim of this work

Although B type allatostatins were isolated from the cricket *G. bimaculatus* by conventional HPLC techniques in our laboratory eight years ago and their inhibition of JH biosynthesis as well as their action as ecdysiostatins were proved by many physiological experiments, the

basic molecular characters of the *G. bimaculatus* $W(X)_6W$-allatostatins, such as the gene structure of the prohormone and the expression patterns of the peptides are still unknown and well worth pursuing. It is a challenge to reveal the relationships between the spatial and temporal expression patterns for the various allatostatin types within one insect species and to correlate their expression with the biosynthesis of the juvenile hormone(s) as well as with other physiological responses which may be affected by the allatostatins. In this work, the following questions were asked:

1) What is the gene structure for the prohormone of *G. bimaculatus* $W(X)_6W$-allatostatins (B type allatostatins)?

2) Where is the expression of the gene localized in various tissues of female and male adult crickets?

3) Which age-dependent changes in the gene expression can be observed in various tissues of adult crickets?

2. Material and Methods

2.1. Material

2.1.1. Rearing of Insects

Mediterranean field crickets *Gryllus bimaculatus* de Geer (Ensifera: Gryllidae) were reared at 27 °C and a relative humidity of 40-60% under 16h light : 8h dark photoperiod. They were fed on excess mixed standard diet for rabbit no. 2021, rat/mouse no. 1311, and cat no. 5031 (Altromin GmbH, Lage). Additionally excess water (Lorenz et al., 1997b) was offered in waterers. Males and females were separated on the day of emergence (0 d) and their age was measured from that time.

2.1.2. Buffers, solutions and media

2.1.2.1. Commonly used buffers and solutions

Fine chemicals were from Merck, Sigma or Roth in p.A. quality, or otherwise as indicated.

10x TBE buffer	890 mM Tris (pH 8.35) 890 mM boric acid, 25 mM EDTA
20x SSC	3 M NaCl (pH 7.0) 0.3 M Na-citrate
Ringer	86 mM NaCl 5.4 mM KCl 3 mM $CaCl_2$
DEPC-water	Milli-Q water was incubated over night with 0.1% (v/v) DEPC (diethyl pyrocarbonate) at 37°C under shaking at 50 rpm, and then autoclaved.

2.1.2.2. Solutions for small-scale preparation of plasmid DNA

Solution I	50 mM glucose 25 mM Tris-Cl (pH 8.0) 10 mM EDTA (pH 8.0)
Solution II	0.2 N NaOH 1% (w/v) SDS
Solution III	3 M sodium acetate (pH 5.2)

2.1.2.3. Buffers and solutions for blotting and hybridization

Depurination solution	0.25 M HCl
Denaturation solution	1.5 M NaCl 0.5 M NaOH
Neutralization solution	1.5 M NaCl 0.5 M Tris-Cl (pH 7.5)
Maleic acid buffer	0.1 M Maleic acid 0.15 M NaCl adjusted to pH 7.5 with solid NaOH and autoclaved

Blocking stock solution (10x)

10% (w/v) blocking reagent (Roche Applied Science, Mannheim) in maleic acid buffer. Solution was melted to 60°C under stirring and then autoclaved

Standard hybridization buffer with formamide

5x SSC (75 mM NaCl, 7.5 mM Na-citrate pH 7.0)
50% (v/v) formamide, deionized
0.1% (w/v) N-lauroylsarcosine

	0.02% (w/v) SDS 2% (v/v) blocking stock solution
Blocking solution (1x)	10% (v/v) blocking stock solution in maleic acid buffer
Washing buffer	3% (v/v) Tween 20 in maleic acid buffer
BCIP stock	50 mg/ml BCIP (Roche Applied Science, Mannheim) in 100% dimethylformamide
NBT stock	100 mg/ml NBT in 70% (v/v) dimethylformamide/ddH_20
Detection buffer	0.1 M Tris-Cl (pH 9.5) 0.1 M NaCl 0.05 M $MgCl_2$

2.1.2.4. Buffers and solutions for RT *in situ* PCR

10x PBS	1.3 M NaCl 0.07 M Na_2HPO_4 0.03 M NaH_2PO_4
Fixation buffer:	1x PBS 4% (v/v) formaldehyde
Proteinase K buffer	0.1 M Tris-HCl (pH 7.5) 0.05 M EDTA
10x buffer 1	1 M Tris-Cl (pH 7.5) 1.5 M NaCl
10x buffer 2	1M Tris-Cl (pH 9.5) 1M NaCl
Blocking solution	1% (w/v) blocking reagent in 1x buffer 1

Colour –substrate solution	350 µl BCIP stock 450 µl NBT stock in 100 ml detection buffer

2.1.2.5. Media and plates

Luria-Bertani-Medium (LB)	10 g pepton 5 g yeast extract 10 g NaCl pH adjusted to 7.0 with NaOH, H_2O added to 1 liter and autoclaved
LB-agar	LB medium containing 1.5% (w/v) agar, autoclaved
IPTG stock	100 mM IPTG (Diagonal, Münster) in ddH_2O
X-gal stock	20 mg/ml X-gal (Diagonal, Münster) in dimethylformamide
Ampicillin	100 mg/ml ampicillin, sodium salt (Promega, Mannheim) in ddH_2O
LB-plates	Freshly autoclaved LB-agar was cooled to 50°C before adding ampicillin to a final concentration of 100 µg/ml. 30 ml were poured into plastic petri dishes (85 mm) and hardened by cooling down. 100µl IPTG stock and 50 µl X-gal stock were spread over the surface of the plates and absorbed prior to use

2.1.3. Bacterial strain

JM109 (Promega, Mannheim) *e14-(McrA-) recA1 endA1 gyrA96 thi-1 hsdR17* (rK- mK+) *supE44 relA1 D(lac-proAB)*[F' *traD36 proAB lacI^{q}ZDM15*]

2.1.4. Primers

UPM 5′ -CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAAC GCAGAGT-3′

UPS 5′ -CTAATACGACTCACTATAGGGC-3′

NUP 5′ -AAGCAGTGGTATCAACGCAGAGT-3′

F25 5′-TGAGCAAGTTCAGCCTGGTTAAGT-3′

F 5′-TGAGCAAGTTCAGCCTGG-3′

MB1r3 5′-CGTTGAGGTCCTGCCA-3

B1-2r 5′-CCTTGAGGTCCTGCCA-3

WB6f 5′-GCCTGGAACAACCTGGGCT-3′

WB6f2 5′-GGGCAAGCGCGCCTGGAACAAC-3′

WB3f2 5′-TGGCGCGACCTCAGCGGCGGCA-3′

WB6r 5′- GGAGCCCAGGTTGTTCCA-3′

WB6r2 5′-CCACGCGGAGCCCAGGTTGTTC-3′

WB3r2 5′-AGCCGCCGCTGAGGTCGCGCCA-3′

HB1f 5′-GCTGGCAGGATCTGAACGGC-3′

WJF2 5′- AAAGCCCTCTCTGCTCTGTGCGAAGTTGCT -3′

WUR2 5′- CAGTAATCATTTACAAGCATTCATGATG -3′

WUR3 5′-TGATGCATTTGATCATTGTACATTAATAGCGCGCGTTGC-3

WUR4 5′- ATTGTAGCTTGCGTCCTCGTACATCGGATG -3′

pUC/M13 Reverse 5'-CAGGAAACAGCTATGAC-3'

pUC/M13 Forward 5'-GTTTTCCCAGTCACGAC-3'

All primers were synthesized by MWG-Biotech AG (Ebersberg).

2.2. Methods

2.2.1. PCR (Polymerase Chain Reaction)

The polymerase chain reaction (PCR) was carried out in a DNA Thermal cycler (PTC-200 Peltier Thermal Cycler; Biozym, Hess. Oldendorf) under standard conditions containing 1-5 ng dsDNA, ssDNA or 100 ng plasmid DNA as a template, 1 µM of each primer, 200 µM of each dNTP and 5 units *Taq* DNA polymerase (MBI Fermentas, St. Leon-Rot), 10 mM Tris-

HCl, pH 8.8, 50 mM KCl, 0.8% nonidet P40, 1.5 mM $MgCl_2$, in a total volume of 50 µl. $MgCl_2$ concentration and the annealing temperature were varied depending on the particular reaction. DMSO was occasionally used in order to get specific amplification.
The cDNA library (Meyering-Vos et al., 2001) was used as a template in combination with the primers F25 and primer B1-2r by running a temperature profile consisting of a predenaturation at 95° C for 5 min, then followed by 5 cycles of a touchdown program of denaturation at 94°C for 1 min, initially annealing for 45 sec at 68° C, decreasing 1°C in each of the 4 following cycles and an extension at 72°C for 1 min. Another 30 polymerisation cycles of denaturation at 94 °C for 1 min, annealing at 60°C for 45 sec, extension at 72°C for 1 min followed and a final extension for 10 min at 72°C.
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The PCR with the 3`RACE reaction as a template and specific primers WB6f and the universal primer UPM has been done with a temperature profile starting with predenaturation at 95 °C for 5 min, followed by 10 cycles of a touchdown program with the denaturation step at 94°C for 1 min, annealing for 45 sec initially at 68°C in the first cycle and lowered 1°C in each of the 9 following cycles in combination with an extension at 72°C for 1 min. Another 30 polymerisation cycles of denaturation at 94 °C for 1 min, annealing at 58°C for 45 sec, extension at 72°C for 1 min and a final extension for 10 min at 72°C followed.

2.2.2. Preparation of plasmid DNA

2.2.2.1. Standard method

The alkali lysis method of Sambrook et al. (1989) was modified for small-scale preparation of plasmid DNA.
1.5 ml of an overnight culture of *E.coli* JM109 was collected by centrifugation at 8000 g for 1 min. The pellet was resuspended in 250 µl solution I, lysed under alkaline conditions with 250 µl solution II for 5 min at room temperature, the lysate was neutralized and adjusted to high-salt binding condition with 350 µl solution III. Afterwards the solution was centrifuged at maximum speed for 10 min, till a compact white pellet was formed. The supernatant was transferred to a new tube, participated by adding equal volume of iso-propanol. After centrifugation for 10 min at top speed, the supernatant was discarded and the pellet was air-

dried for 10 min. 40 µl ddH_2O was added to the tube to dissolve the plasmid DNA and the solution was kept at 4°C .

2.2.2.2. Purification of plasmids for sequencing

The QIAprep Spin Miniprep kit (Quiagen) was used to purify plasmid DNA. 5 ml of an overnight culture of *E. coli* JM109 was collected by centrifugation in aliquots of 2 ml in a centrifuge type 5415C (Eppendorf, Hamburg) at 8000 g for 1 min. The pellet was resuspended, lysed and purified according to the manufacturers instructions. The plasmid was eluted from the column with 50 µl of elution buffer (10 mM Tris, pH 8.5) and stored at –20°C.

2.2.3. Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualize and isolate DNA molecules following PCR amplification or restriction digestion. Agarose (0.8-2%) was dissolved in 0.5x TBE buffer by heating in a microwave. After cooling, 2 ml of a 10 mg/ml ethidium bromide solution was added per 100 ml gel and the gel was poured. Gels were routinely run at 80-100 V for 1-2 hours, depending on the size of the examined DNA fragment or on the degree of band separation required. For determination of fragment size the 100 bp (100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp) or 1kbp DNA ladder (250, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, 10000bp) (Diagonal, Münster) was used.

2.2.4. DNA purification from agarose gel

The GFX PCR DNA and Gel Band Purification kit (Amersham, Freiburg) was used to purify DNA from agarose gel. The DNA fragments were cut out from the agarose gel on a UV-FLÄCHENSTRAHLER (Konrad Benda) by illumination at 322 nm. A maximum of 300 mg agarose gel was applied to one centrifuge column of the kit. The solution and purification of the DNA fragments was done according to the protocol of the manufacturer. For elution of the DNA from the centrifuge columns 50 µl of elution buffer was applied. The elutant was stored at –20°C.

2.2.5. Cloning of PCR products

For cloning of PCR products the pGEM-T Easy Vector System I kit (Promega, Mannheim) was used. The reaction mixture consisted of 20-100 ng (~3µl) purified PCR product, 50 ng pGEM-T easy vector, 1 µl (3U) T4 DNA ligase and 5 µl 2x rapid ligation buffer. The solution was mixed well and incubated at 25°C for 1 hour. 2 µl of the ligation mixture was used for transformation.

2.2.6. Preparation and transformation of competent *E. coli* cells

2.2.6.1. Preparation of competent cells

The method of Sambrook et al. (1989) was modified to prepare and transform the competent cells.

E. coli JM 109 (Promega, Mannheim) were streaked on LB agar and grown 16-20 hours at 37°C. A single colony of *E. coli* JM109 was picked and transferred into 100 ml of LB medium in a 1-liter flask. The culture was incubated for ~3 hours at 37°C with vigorous shaking (300 rpm) until the OD_{550} was between 0.45-0.55. Then the culture was transferred to sterile, ice-cold 50 ml centrifuge tubes and cooled to 0°C by storing the tubes on ice for 10 min. After the cells were recovered by centrifugation at 1300 g in a centrifuge type 5415C (Eppendorf, Hamburg) for 10 min at 4°C, the LB media was decanted and the tubes were kept in an inverted position for 1 min to allow the last traces of media to drain away. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice. The centrifugation and decantation was repeated. Then each pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$/15% (v/v) glycerol and stored on ice. 200 µl of this suspension was transferred to a 1.5 ml microfuge tube, then kept at –80°C.

2.2.6.2. Transformation

The tubes containing the competent cells were kept on ice for 10 min to thaw the cells. Then the plasmid DNA or ligation mixture was added to the tubes and the solution was gently swirled several times to mix the contents. The volume of plasmid DNA should not exceed 5% of the competent cells. After the tubes have been kept on ice for 30 min they were placed in a

preheated 42°C water bath for exactly 60 sec without shaking. Then they were rapidly transferred to an ice bath and kept for 1-2 min. 800 µl of LB medium was added to each tube and the cultures were incubated for 45 min at 37°C by shaking at 150 rpm to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. 50-200 µl of the transformed cells were transferred onto LB agar plates containing appropriate antibiotic and they were gently spread over the surface of the agar plate with a sterile bent glass rod. The plates were stored at room temperature until the liquid has been absorbed and then they were incubated in an inverted position at 37°C for 12-16 hours, until colonies appeared.

2.2.7. Restriction digestion

The restriction enzymes are bacterial enzymes that recognize specific 4-8 base pair nucleotide sequences called restriction sites, and then cleave both DNA strands at this site.

To digest the template DNA with the adequate restriction enzyme, 100 ng - 5 µg DNA, 1-5 U/µg DNA restriction enzyme and 10% (v/v) enzyme specific restriction buffer was mixed and incubated 1-3 hours, depending on degree of digestion required. EcoR I (Promega, Mannheim) was used to digest the plasmid derived from the pGEM-T Easy vector in order to check the correct insertion.

2.2.8. Synthesis of DIG- labelled cRNA probe

The DIG RNA Labeling Kit (SP6/T7) (Roche, Mannheim) was used for the synthesis of DIG-labelled cRNA probe.

The DNA template of cRNA probe synthesis was prepared by PCR, using plasmid, which was constructed by inserting PCR product encoding *Grybi*–AST B (nt 1-199) into pGEM–T Easy vector as a template. A PCR product of 509 bp, amplified with the pUC/M13 reverse and forward primer, was purified from the agarose gel after electrophoresis. 1 µg of purified DNA was used as a template and the volume adjusted to 13 µl with DEPC–water in a RNase–free tube. 2 µl of NTP labelling mixture, 2 µl of transcription buffer, 1 µl RNase inhibitor and 2 µl of RNA Polymerase SP6 (or T7) were added. Then the tube was centrifuged briefly, incubated at 37°C for 2 hours and finally the reaction was stopped by adding 2 µl of 0.2 M

EDTA. The same method was used for synthesis of cRNA probe of actin of *G. bimaculatus* according to the sequence submitted to GenBank (Accession no. AB087882).

2.2.9. Extraction of total RNA

The peqGOLD Trifast kit (Peqlab, Erlangen) was used to extract total RNA from *G. bimaculatus*. The various tissues from *G. bimaculatus* adults of different ages were dissected under modified Ringer (Lorenz et al., 1997b) and frozen immediately under liquid nitrogen. The tissue was ground to a fine powder in a precooled mortar and pestle under liquid nitrogen and 50-100 mg were transferred to a 1.5 ml Eppendorf tube. Lysis, extraction and purification of the RNA was done according to the instruction manual of the kit. The pure RNA pellet was air dried for 5 min and then dissolved in 100 µl RNase-free water. Concentration was determined spectrophotometrically.

2.2.10. Extraction of mRNA

The Oligotex mRNA Mini kit was used to extract mRNA from *G. bimaculatus* total RNA. 1 mg total RNA was pipetted into a RNase-free 1.5 ml microcentrifuge tube and the volume was adjusted to 250 µl with RNase-free water. Further purification steps have been done according to the instructions of the manufacturer. For elution 50 µl hot (70°C) buffer OEB was applied to the column, pipetted up and down 3 or 4 times to resuspend the resin and then centrifuged for 1 min at 15000 g in a centrifuge 5415C (Eppendorf, Hamburg). This step was repeated, to ensure maximal yield. For digestion of traces of genomic DNA 1 µg of mRNA was incubated in presence of 10 U DNase (Promega, Mannheim) at 37°C for 30 min, followed by a denaturation at 75°C for 30 min and an extraction of the DNase by chloroform. The concentration was determined spectrophotometrically and the purified mRNA stored at –80°C until use.

2.2.11. RACE (Rapid Amplification of cDNA Ends)

For the RACE reactions the SMART™ RACE cDNA Amplification Kit was used.

1 µg mRNA was used for each reaction. The contents were mixed in separate 0.5 ml tubes as follows:

5´ RACE –Ready cDNA		3´ RACE –Ready cDNA	
1-3 µl	mRNA sample	1-3 µl	mRNA sample
1 µl	5´CDS primer	1 µl	3´CDS primer
1 µl	SMART II oligo		

The final volume was adjusted to 5 µl with DEPC-water and the solutions were mixed by briefly spinning. After an incubation period of 2 min at 70°C, the tubes were put on ice immediately for 2 min and the reaction complemented by 2 µl 5x first–strand buffer, 1 µl DTT (20 mM), 1 µl dNTP mix (10 mM) and 1 µl PowerScript Reverse Transcriptase to give a total volume of 10 µl. The tubes were centrifuged briefly and then incubated at 42°C for 1.5 hours in an air incubator. The first–strand reactions were diluted with 250 µl Tricine-EDTA buffer and 2 µl was used as template for each PCR with the primer Upm and a gene-specific primer.

2.2.12. Extraction of genomic DNA

For extraction of genomic DNA from tissues of the whole animal of *G. bimaculatus* the QIAGEN Genomic-tip 500/G kit (Qiagen, Hilden) was used.

The cuticula of *G. bimaculatus* adults was removed and the tissues immediately frozen in liquid nitrogen and ground to a fine powder with a precooled mortar and pestle. 1 g ground tissue was transferred to a 50 ml screw-cap tube and 19 ml buffer G2 was added and complemented with 72 µl of RNase A (Roche, Mannheim) to give a final concentration of 1.8 µg/ml to the tube. The mixture was incubated at 37°C for 30 min. 800 µl Proteinase K stock solution (20 mg/ml in H_2O) was supplied before the incubation at 50°C for 2 hours.

The sample was centrifuged at 2000 g in a centrifuge type 5415C (Eppendorf, Hamburg) for 5 min to get rid of particles, applied to a QUIAGEN Genomic-tip (Quiagen, Hilden), which was equilibrated as recommended and allowed to enter the resin by gravity flow. Washing, elution and precipitation was done as described by the supplier. The pellet was washed with 70% (v/v) ethanol, air dried and dissolved in 200 µl H_2O. The concentration and purity was detected by measuring the absorbance at 260 and 280 nm. The DNA was stored at –20°C.

2.2.13. Southern blot

For each restriction enzyme digestion (or double digestion) 10 µg purified genomic DNA was digested overnight with 30 units of different restriction enzymes such as Apa I, BamHI, ClaI, EcoR I (Promega) or a combination of ClaI and EcoR I, that did not cleave within the known allatostatin cDNA sequence.

A 1% agarose gel not thicker than 4 mm was run following the standard protocol and afterwards stained and photographed. Then the gel was rinsed in distilled water, submerged in 10 volumes of depurination solution and shaked gently on a shaker (GFL 3011, Burgwedel) for 30 min. All following steps have been carried out at room temperature. After rinsing with distilled water, it was submerged in 10 volumes of denaturation solution and shaked gently on a shaker for 20 min at room temperature. This last step was repeated. Addition of 10 volumes of neutralization solution and shaking for 20 min followed. After repeating this step a classical capillary Southern blot was performed by using a Nylon N+ membrane (Amersham, Mannheim) to bind the fragments. After 1 to 4 hours, the paper towels were removed, the membrane was transferred into 300 ml 6x SSC buffer and carefully rinsed for 5 min. The membrane was removed from the 6x SSC and excess fluid was drained away. Then the membrane was put on a dry sheet of Whatman 3MM paper with DNA side upward for a few minutes. The DNA fragments were crosslinked to the membrane by UV irradiation at 254 nm at 120 J/cm^2 in a Biolink BLX 254 UV crosslinker (Vilbert Lourmat, Marne-La Vallee, France)..

2.2.14. RNA dot blot

The dot-blot manifold containing 64 wells was cleaned in 0.1 M NaOH for 30 min, then rinsed in DEPC-water for several times. A piece of nylon N+-membrane (Amersham, Mannheim) and a piece of 3MM filter paper were cut to appropriate size and prewetted in DEPC-water. The membrane and the 3MM filter paper were inserted into the manifold and the apparatus was assembled. For each sample 100 ng mRNA were diluted into 50 µl DEPC-water, mixed with 30 µl 20x SSC and 20 µl 37% (v/v) formaldehyde, heated to 65°C for 15 min and then chilled on ice. After the vacuum was turned to each well 500 µl 20x SSC was added and sucked through. The sample was applied and after going through, 500 µl 20x SSC was added to the well. These steps were repeated to finish loading of all the samples. The

manifold was disassembled and the membrane was removed, soaked into 100 ml 6x SSC buffer and carefully rinsed for 5 min. Then excess fluid was drained away and the membrane was put on a dry sheet of Whatman 3MM paper with RNA side upward for a few minutes. Finally, the membrane was cross-linked by UV irradiation at 254 nm at 120 J/cm^2 in a Biolink BLX 254 UV crosslinker (Vilber Lourmat, Marne-La-Vallee, France).

2.2.15. Hybridization and detection

20 ml standard hybridization buffer was prewarmed to 60°C and the membrane of the southern blot or from the dot Northern blot was incubated for 30 min with gentle agitation. 1 µg DIG-labeled cRNA probe was denatured by boiling for 5 minutes and rapidly cooled on ice for 5 min, then added to 3 ml prewarmed standard hybridization buffer. Prehybridization, hybridization and detection by the colourimetric method has been done according to the protocol of the DIG DNA labelling and detection kit (Roche Applied Science, Mannheim). The hybridization has been performed at 60°C by an overnight incubation.
When the desired band intensities were achieved, the reaction was stopped by washing the membrane in 50 ml water for 5 min. The results were documented by photography with the Image Master VDS system (Amersham, Freiburg) and the intensity of the bands was calculated with the Image Master 1D Database software.

For the chemoluminescent detection the membrane was placed with mRNA side facing up on a development folder and 2 ml diluted CSPD solution (1:100 in detection buffer) was applied on. The membrane was covered with the second sheet of the folder immediately to spread over the substrate evenly and without airbubbles over, then incubated for 5 min at 15-25°C. The excess liquid was squeezed and the development folder was sealed. The damp membrane was incubated at 37°C for 15 min to enhance the luminescent reaction, then exposed to a Kodak X-Omat AR X-ray film (Sigma, Taufkirchen) for 5-25 min at 15-25°C. Multiple exposures were taken to achieve the desired signal strength.

The results were documented by photography with the Image Master VDS system (Amersham, Freiburg) and the intensity of the bands or dots was calculated by the Image Master Dot Blot Database Software. The integrated optical density (IOD) of allatostatin type B and actin for the respective tissues from 3 day old adults were calibrated as standards. The levels of allatostatin type B expression were normalized by comparing the IOD of allatostatin

type B relative to the IOD of actin of the same matched sample. All results are represented as means ∀ SD of measurements as indicated in the figure legends. The data were not further treated by statistics, because the values are normalized and relatively expressed.

2.2.16. RT-PCR (Reverse transcriptase-polymerase chain reaction)

The Titanium one-step RT-PCR kit was used for one-step RT-PCR as follows. A master mix was prepared for 10 reactions: 10 µl 10x one-step buffer, 2 µl 50x dNTP mix, 1 µl recombinant RNase inhibitor, 50 µl thermostabilizing reagent, 20 µl GC-melt, 2 µl Oligo(dT) primer, 1 µl forward primer WJF2 (20 µM), 1 µl reverse primer WUR3 (20 µM), 1 µl DEPC-water, 2 µl 50x RT-Titanium *Taq* enzyme mix in a total volume of 90 µl. 1 µl of 20 ng/µl mRNA of different tissues was added to an aliquot of 9 µl master mix and centrifuged briefly. The PTC-200 Peltier Thermal Cycler (Biozym, Hess. Oldendorf) was used to run the RT-PCR temperature profile. The reaction started by an incubation for reverse transcription for 1 h at 50°C, followed by 5 min denaturation at 94°C, 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C and 1 min elongation at 72°C, finished by one cycle of final extension 2 min at 72°C. As a control for genomic DNA contamination, a PCR in the presence of *Taq* DNA polymerase (MBI Fermentas, St.Leon-Rot) has been done using the same amount of template mRNA as in the RT-PCR reaction and the temperature profile varied by leaving out the first cycle.

2.2.17. RT *in situ* PCR

2.2.17.1. Fixation, dehydration, and embedding

The tissues were dissected from 1 day old *G. bimaculatus* adults under Ringer solution, immediately fixed in a modified fixation buffer, which was renewed every 30 min for 4 times. After the tissues have been kept at 4^{o}C for 2 days, they were dehydrated using a graded ethanol series of 30, 50, 70, 85, 95, 100% (v/v) each incubated 3x for 30 min with the exception of the third step of 85%, which has been performed overnight.

Another series of fixation followed by using ethanol : Roticlear (Roth, Karlsruhe) in the ratio of 3:1; 1:1, 1:3 each incubated 3x for 30 min and then three times with 100% Roticlear for 60 min. The tissues in Roticlear were warmed in a oven at 55°C for 4 hours, meanwhile 10-20

paraplast chips (paraplast embedding media Paraplast plus; Sigma, Taufkirchen) were added every 1 hour. When all the chips were melted, half of the paraplast-Roticlear mixture, in which the tissues were immersed, was poured out and fresh molten paraplast was poured in. This was repeated once and then all the paraplast-Roticlear mixture was poured out and new fresh molten paraffin was poured in. After an overnight incubation at 55°C, the paraplast was replaced with fresh molten paraplast every 4 hours for 2 days. Tissue blocks were cast in plastic disposable frames. A heating block was used at 70°C to keep the paraplast molten while the tissue was correctly oriented.

2.2.17.2. Sectioning and mounting to slide

The tissue blocks were trimmed to a rectangular cutting face, then sectioned at 10 µm. 750 µl of DEPC-H_2O were dropped onto the slides and the cut ribbons were floated on the water.
The slides were heated at 42°C for 5 min on a warming tray to extend tissue sections, then the water was removed by wiping with 3MM filter paper and the slides were dried for 48 hours on a warming tray at 42°C to ensure adhesion.

2.2.17.3. RT *in situ* PCR and detection

The slides were put into Roticlear and incubated at 50°C for 30 min to remove the paraffin. The sections were incubated two times for 2 min in 100% ethanol, then air-dried in a dust free chamber at room temperature. After they had been treated with 200 ml Proteinase K solution (2 µg/ml) at 37°C for 5-90 min depending on the tissue, the slides were rinsed twice in DEPC water for 5 min, incubated at 95°C for 2 min to inactivate Proteinase K activity and submerged in 200 ml 0.1 M triethanolamine solution supplemented with of 1.0 ml acetic anhydride. The slides were incubated in triethanolamine solution for 10 min, rinsed for 5 min in DEPC water and incubated in 100% ethanol for 5 min followed by drying in a dust-free chamber at room temperature. To digest the genomic DNA 40 µl of digestion solution composed of 4 µl DNase I buffer, 2 µl RNase free DNase I with 10 U/µl (Promega, Mannheim), RNase inhibitor with 40 U/µl (Promega, Mannheim) and 33 µl of DEPC water were applied to each slide. A control has been done by leaving out the RNase inhibitor but additionally using 10 µl of RNase to digest the RNA. The slides were covered with cover glasses and placed in a humid incubator at 37°C overnight. Then they were washed twice for 5 min in DEPC -water and left for 2 min at 95°C to inactivate DNase I activity. For one–step

RT-PCR the reaction components delivered with the Tth DNA polymerase (Promega, Mannheim) were used: 10 µl of $MnCl_2$, 10 µl of reverse transcriptase buffer (Promega, Mannheim), 8 µl $MgCl_2$, 8 µl chelate buffer, 1 µl Tth DNA polymerase with 5 U/µl - and additionally 2 µl dNTP mix 10 mM each, 3 µl forward primer WJF2 and reverse primer WUR3 at 10 pmol/µl, respectively, and 0.5 µl 200 µM dig-dUTP (Roche, Mannheim) were mixed. 54.5 µl of DEPC water was added to yield a total volume of 100 µl. 40 µl of the reaction mix was applied to each section, then the slides were covered with *in situ* frames and the *in situ* RT-PCR was performed with PTC-100 Peltier Thermal Cycler (Biozym, Hess. Oldendorf) with the temperature profile of 45 min at 60°C, 3 min of 94°C, 30 cycles of 45 sec at 94°C, 1 min at 60°C and 1 min at 72°C. The slides were washed in 0.2x SSC/0.2% BSA at 60°C for 15 min, and incubated in blocking buffer for 30 min The anti-DIG-AP conjugate (Roche, Mannheim) was diluted to 750 mU/ml (1:1000) in blocking solution and after 200 µl were added to each slide they were incubated in a humid chamber at room temperature overnight. After the slides have been rinsed twice for 10 min in buffer 1 and three times for 5 min in buffer 2 they were incubated in colour–substrate solution at 30°C in the dark and observed every 30 min. When brown or blue colour developed, the slides were washed in water three times for 5 min each, in 70% ethanol for 30 sec and finally in 100% ethanol for 15 sec. A drying step at room temperature in a dust-free chamber followed and afterwards 150 µl of Aquatex mounting medium was added to each slide and the cover glasses were placed very slowly onto each slide. After the extra mounting medium had been removed by wiping with 3MM paper, the slides were placed on desktop for one day and could then be examined with the Leitz Diaplan microscope with a Wild Leitz MPS 46 photoautomate or with digital camera (CoolSNAP RS Photometrics).

2.2.18. Sequence comparison and analysis

Allatostatin B type peptide sequences were used for comparison from various organisms (for references see Table. 1). The software package of the Genetics Computer Group (GCG, version 10.1) of the University of Wisconsin was used for sequences alignment analysis, which was carried out with PILEUP.

3. Results

3.1. Identification of the 3´cDNA sequence of *Grybi*-AS B gene

3.1.1. Isolation of the partial cDNA sequence of *Grybi*-AS B gene

The PCR and RACE methods were used for isolation of the *Grybi*-AS B gene. The polymerase chain reaction (PCR), invented and developed by Karry Mullis (Mullis et al., 1986), is a highly sensitive method for the detection of low amount of target sequence. In general, PCR amplification of target molecular requires two sequence-specific primers that flank the region of the sequence to be amplified (Saiki et al., 1985, 1989). However, to amplify and characterize a region of unknown sequences, this requirement imposes a severe limitation (Loh et al., 1989). Based on PCR technique, the methodology of RACE (Rapid Amplification of cDNA Ends), which was devised by Michael et al. (1988), offers possible solutions to this problem. Rapid amplification of cDNA Ends is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequences at either the 3´or 5'-end of the mRNA. This methodology of amplification with single-sides specificity has been described by others as "one-sided " PCR (Ohara et al., 1989) or "anchored PCR"(Loh et al., 1989). It is a simple and efficient cDNA cloning strategy that overcomes many of the difficulties encountered in obtaining full-length cDNA clones of low-abundance mRNAs (Michael et al., 1988).

A random primer cDNA library (Meyering-Vos et al., 2001) was used as a template for screening by PCR. To improve specificity, the touchdown method was employed (Don et al., 1992). With the vector-derived primer F25 and primer B1-2r, a PCR product of 272 bp was yielded after the amplification (Fig. 1). After sequencing, this fragment proved to contain a core region of the *Grybi* -AS B prehormone precursor (corresponding to nt 1-199) excluding the sequence of the cDNA library arm.

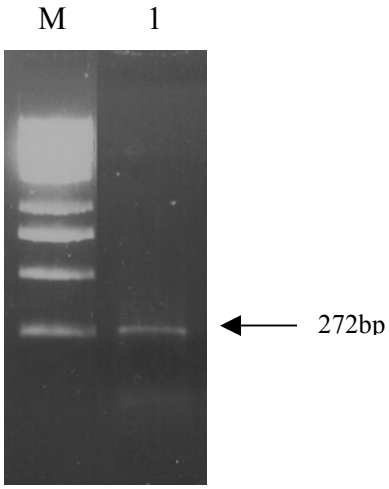


Fig.1 Agarose gel electrophoresis of the PCR product using random primer cDNA library as a template and the primer F25 and primer B1-2r. M: 1 kbp marker; 1: separation of the PCR product. Length of the fragment is given at the right side.

According to the sequence information of this fragment, gene-specific primers were designed for 3`RACE. With the sequence-specific primer WB6f and the universal primer UPM, a PCR product of about 550 bp was amplified (Fig. 2 A). After purification from agarose gel, this fragment was used as a template for nested PCR with the same program using primers NUP and HB1f. After amplification, three bands (470 bp, 434 bp and 398 bp) were yielded (Fig. 2 B) according to the replicates of *Grybi*-AS B1. The whole procedure of 3´cDNA cloning is summarized in Fig. 3.

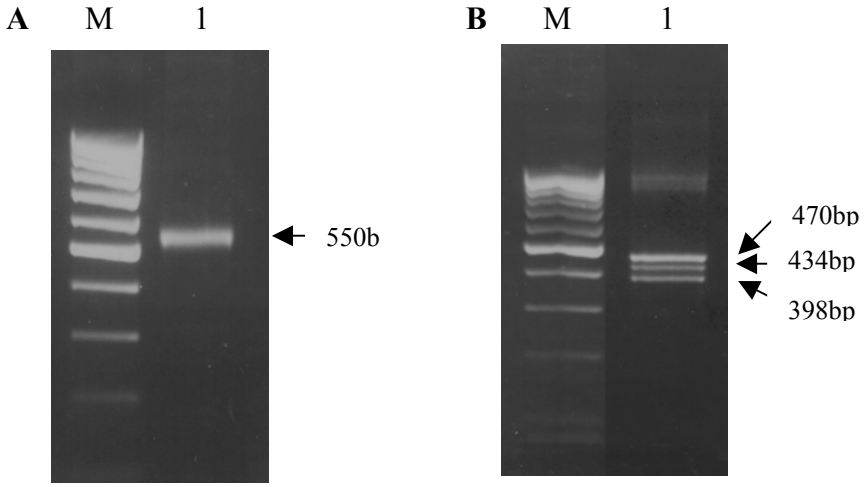


Fig. 2 Electrophoresis of the 3´- RACE products. The 100 bp marker (M) is shown on the left of each electrophoresis. The PCR products were amplified with the primers UPM and WB6F (A-1) or in a nested PCR reaction with the primers NUP and HB1f (B-1).

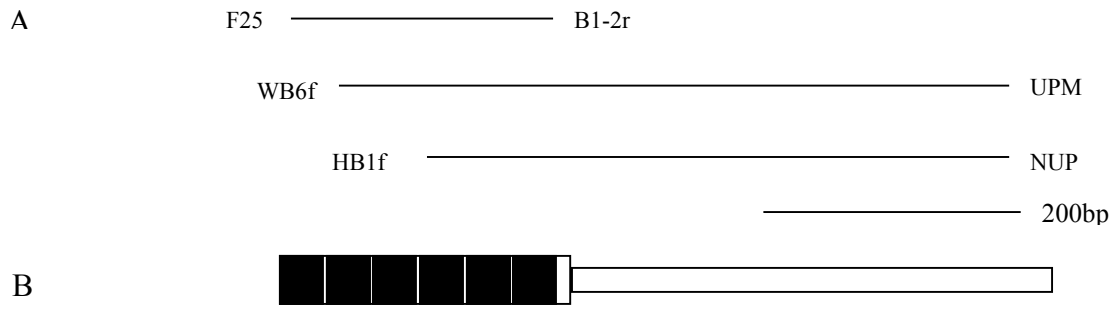


Fig. 3 Schematic representation of the partial cDNA sequence of *Grybi*-AS B gene. (A) Position of the cDNA clones. The upper clone was amplified from the random primer cDNA library with primers F25 and B1-2r, the middle clone was amplified with primers UPM and WB6f, the lower clone was amplified by nested PCR with primers NUP and HB1f. (B) The 3´cDNA sequence of *Grybi*-AS B gene. The 3´cDNA sequence of *Grybi*-AS B gene is given as bars, being broad for the coding region and small for the noncoding region. Black blocks represent sequences encoding *Grybi*-AS Bs.

Combination of the data yielded a 535 bp 3´cDNA sequence (Fig. 4) .The coding sequence is terminated with the UGA stop codon (nt 256). Duplicated stop condons of UAG (nt 307) which were preferentially used in genes with a high GC content (Rice et al., 1994) are found downstream nearby as another putative translation termination signals. The two AAUAAA motifs, which represent a putative polyadenylation signal, were found (nt 483, nt 505) in the 3´untranslated region of twenty eight or six nucleotides upstream from the polyadenylation site.

3.1.2. Verification of the 3´cDNA sequence of *Grybi*-AS B gene

Comparison of the putative translation product of this sequence with five allatotatin B type peptides isolated from the cricket by methanolic extraction and HPLC-purification (Lorenz et al., 1995a, 1999) indicates that this sequence represents the 3´end of *G. bimaculatus* allatostatin B (*Grybi*-AS B) cDNA. The identified 3´ cDNA fragment encodes a putative translation product of 85 amino acids (Fig. 4) with potential dibasic endoproteolytic cleavage sites, which may allow processing into six peptides including three copies of Grybi-AS B 1 (GWQDLNGGWG) and single copies of Grybi-AS B 2 (GWRDLNGGWG), Grybi-AS B 3 (AWRDLSGGWG), and Grybi-AS B 6 (AWNNLGSAWG), respectively. Three of these deduced peptides were previously isolated from cricket brain extracts and were designated as Grybi-AS B 1, Grybi-AS B 2, and Grybi-AS B 3 (Lorenz et al., 1995a). The Grybi-AS B 6

(AWNNLGSAWG) represents a new member of the B type allatostatins. All these prepeptides end with a Gly that affords C-terminal amidation peptidylglycine α-amidation monooxygenase (Bradbury et al., 1982). The α- amidation is essential for the activity of the hormones (Pratt et al., 1991). All of the four peptides contain the W^2W^9 motif that is typical for the allatostatin B type peptides of the $W(X)_6$Wamide family.

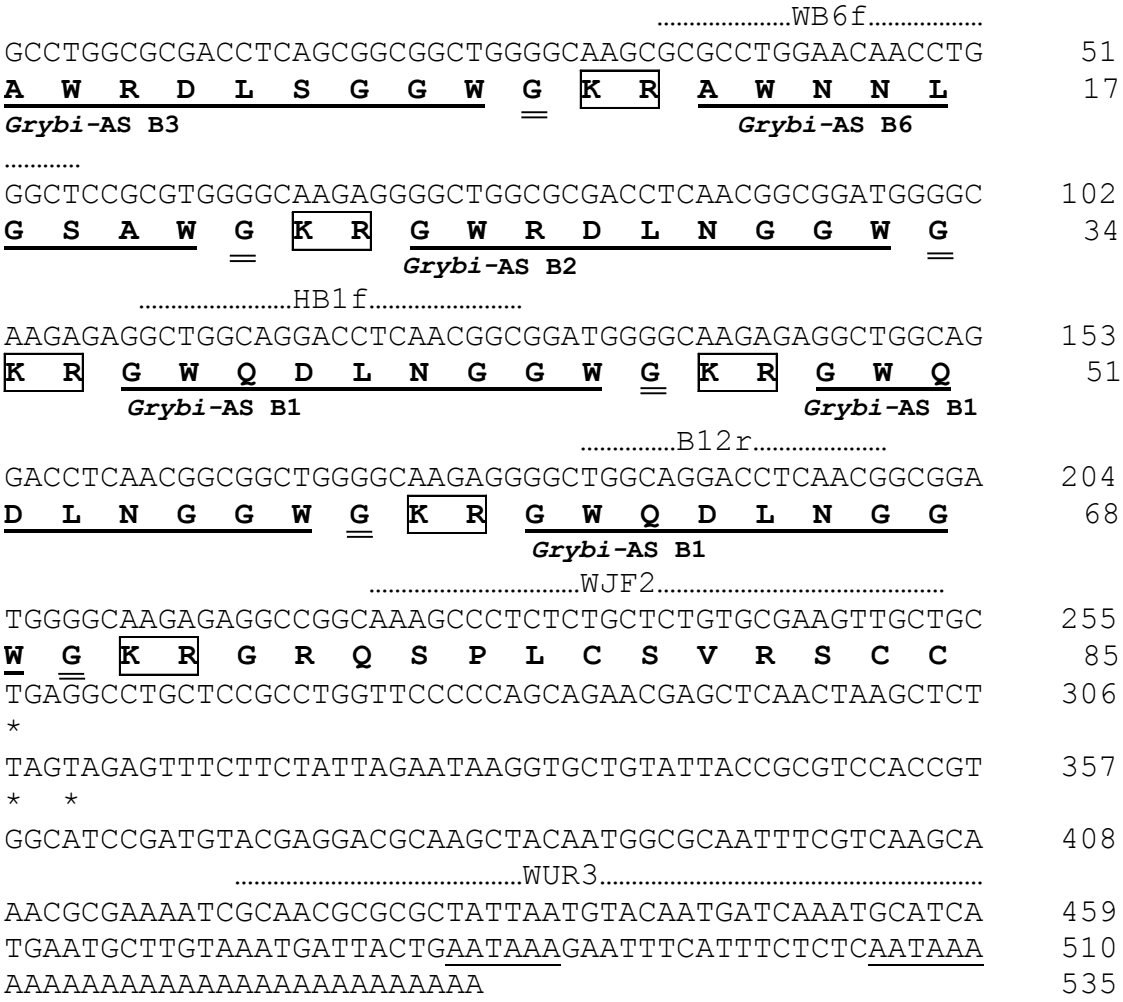


Fig. 4 The 3´cDNA and the deduced amino acid sequence of the 535 bp 3´cDNA sequence. The 3´cDNA sequence is numbered from the first identified nucleotide at the 5´end. The positions of primers used in PCR, RT-PCR, and RT *in situ* PCR are marked with dots, the potential polyadenylation signal is underlined. The deduced amino acid sequence is in boldface and numbered on the right. Potential cleavage sites are boxed and the glycine residues required for α-amidation are double underlined. Allatostatin B type peptides are underlined. * represents stop codon.

The sequences encoding the individual B type allatostatins are high in GC-content and show high homology (Table. 2). The identity of the sequences encoding Grybi-AS B 2 and Grybi-AS B 1, which is present in three replicates, is 93.3 %, whereas *Grybi*-AS B2 and B3 share 90% nucleotide identity. The highest GC-content was found in the sequence of *Grybi*-AS B 3

with 83.3%. GC-contents of the sequences encoding *Grybi*-AS B 1, B 2 and B 3 are 73.3%, 76.6% and 73.3 %, respectively.

Table. 2 Homology and GC-content analysis of the sequences encoding *G. bimaculatus* B type allatostatins

Sequences			GC-Content
	(1)	1 10 20 30	
B1	(1)	GGCTGGCAGGACCTCAACGGCGGATGGGGC	73.3%
B2	(1)	GGCTGGCGCGACCTCAACGGCGGATGGGGC	76.6%
B3	(1)	GCCTGGCGCGACCTCAGCGGCGGCTGGGGC	83.3%
B6	(1)	GCCTGGAACAACCTGGGCTCCGCGTGGGGC	73.3%

3.2. Comparison of *G. bimaculatus* type B allatostatins with other known W(X)$_6$Wamides

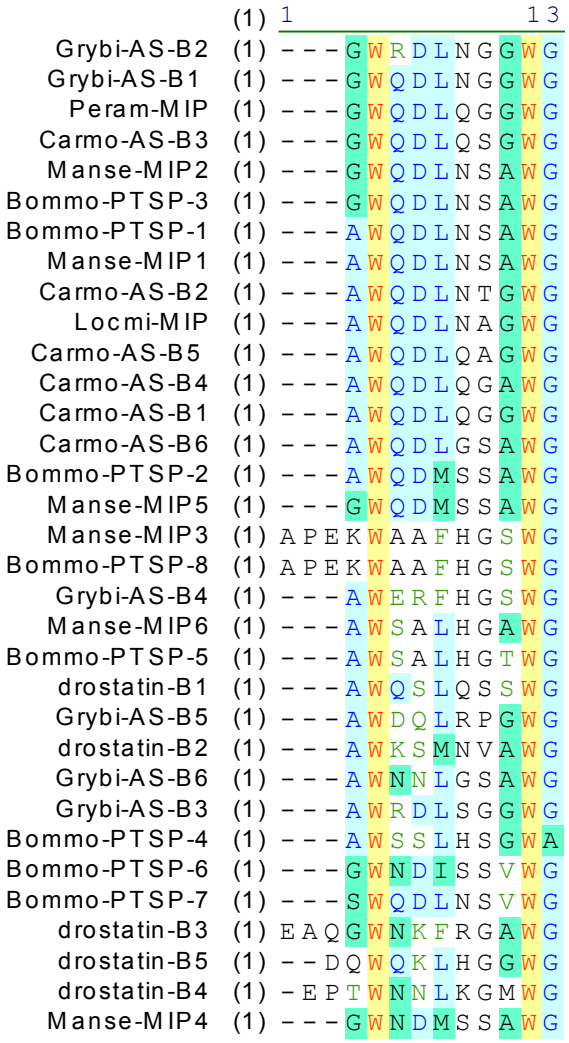


Fig. 5 Homology analysis of W(X)$_6$W-allatostatins.

There are structural similarities between the identified Grybi-AS B type peptides and the $W(X)_6W$ allatostatins from the stick insect *C. morosus*, the silkworm *B.mori,* the fruitfly *D. melanogaster,* the locust *L. migratoria,* the cockroach *P. americana* and the moth *M. sexta* (Fig. 5). The Grybi-AS B 1 and B 2, which show 90% identity, are highly homologous with Carmo-AS B 3 and Peram-MIP, respectively, whereas Grybi-AS B 5, is low in homology to other $W(X)_6W$-allatostatins.

3.3. Southern blot analysis of the B type allatostatin gene

To determine the number of *Grybi*-AS B genes within the *G. bimaculatus* genome, DNA was isolated from individual adults and digested with the different restriction enzymes Apa I, BamHI, ClaI, EcoR I or a combination of ClaI and EcoR I, that did not cleave within the known allatostatin cDNA sequence. After electrophoresis, digested DNA was transferred to a nylon N^+-membrane and probed with DIG-labelled cRNA corresponding to the coding region of *Grybi*-AS B 3 to B 1 (nt 1-199). A single hybridizing band was found with the digestion of ApaI, ClaI or double digestion of ClaI/EcoR I (Fig. 6). This result is indicative of a single copy of the *Grybi*-AS B gene per haploid *G. bimaculatus* genome.

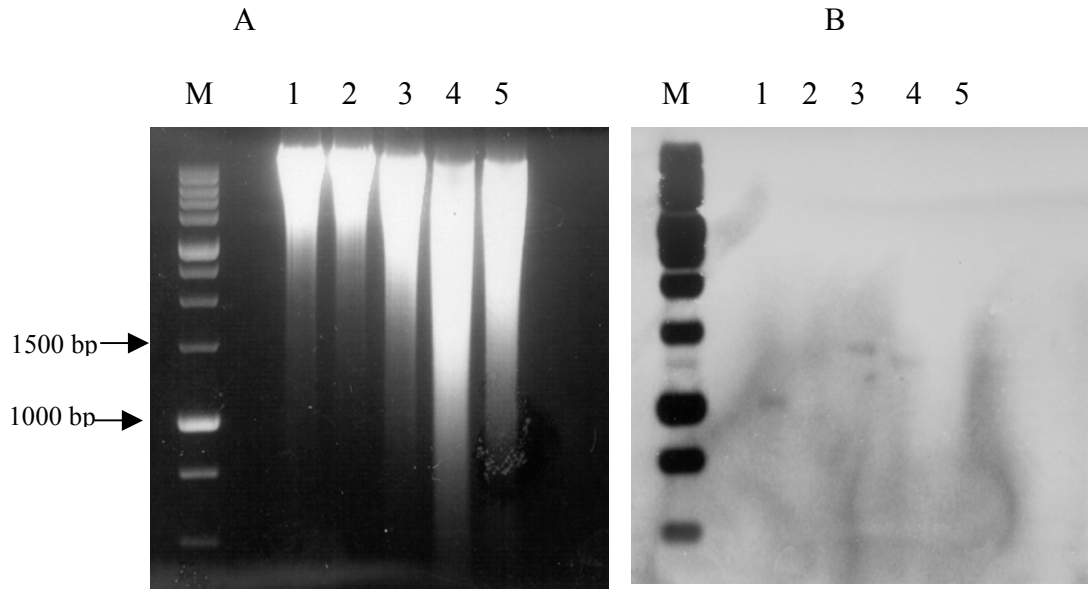


Fig. 6 (A) Agarose gel electrophoresis of the genomic DNA samples (10 µg/lane) digested with the restriction endonucleases ApaI (1), BamHI (2), ClaI (3), ClaI/EcoRI (4), EcoRI (5). (B) Southern blot probed with DIG labelled cRNA (corresponding nt 1 - 199 of Fig. 4). (M) The 1 kbp DNA size marker bands are indicated on the left.

3.4. *Grybi*-AS B type expression

3.4.1. *Grybi*-AS B expression in various tissues of 1 day old females or males

The expression of the *Grybi*-AS B gene was investigated by means of one-step RT-PCR. The mRNA was isolated from tissues of 1 day old virgin females or males, then digested with DNase I and subjected to one-step RT-PCR. For each tissue, amplification yielded a unique product of 236 bp (Fig. 7 A). The same amount of mRNA sample from each tissue was used as template for PCR with *Taq* DNA polymerase using the same cycling profile, but excluding the step of reverse transcription. After amplification with gene–specific primers WJF2 and WUR3, no PCR product could be detected (Fig. 7 B). Thereby, it was proven that the PCR products of 236 bp were amplified from the mRNA templates.

The results of the one-step RT-PCR suggested that the *Grybi*-AS B gene was expressed in brain, suboesophageal ganglion, thoracic ganglion, abdominal ganglion, ovary, caecum and midgut, hindgut, fat body, accessory reproductive glands, and the thoracic muscles of 1d-old adult female crickets and in brain, accessory reproductive glands, and testes of 1d-old adult male crickets. Subsequently, the cycling profile of RT-PCR which yielded the unique PCR product could be used for *in situ* RT-PCR.

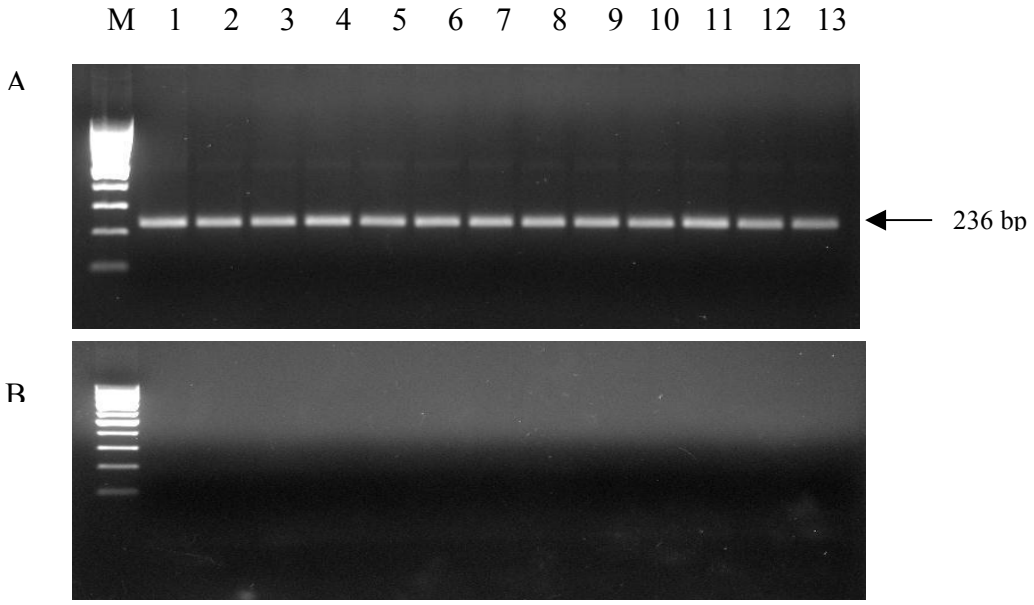


Fig. 7 (A) Agarose gel electrophoresis of the RT-PCR products stained with ethidium bromide. On the left the 100 bp marker was run. The mRNA of brain (1), suboesophageal ganglion (2), thoracic ganglia (3), abdominal ganglia (4), ovary (5), caecum and midgut (6), hindgut (7), fat body (8), accessory reproductive glands (9) and thoracic muscle (10) of 1d-old female adult crickets, and brain (11), accessory reproductive glands (12) and

testes (13) of 1d-old male adult crickets were used as a template. (B) Agarose gel electrophoresis of the control PCR amplification to test the genomic DNA contamination of the matched mRNA samples.

3.4.2. Localization of *Grybi* - AS B expression in various tissues of 1 day old virgin females

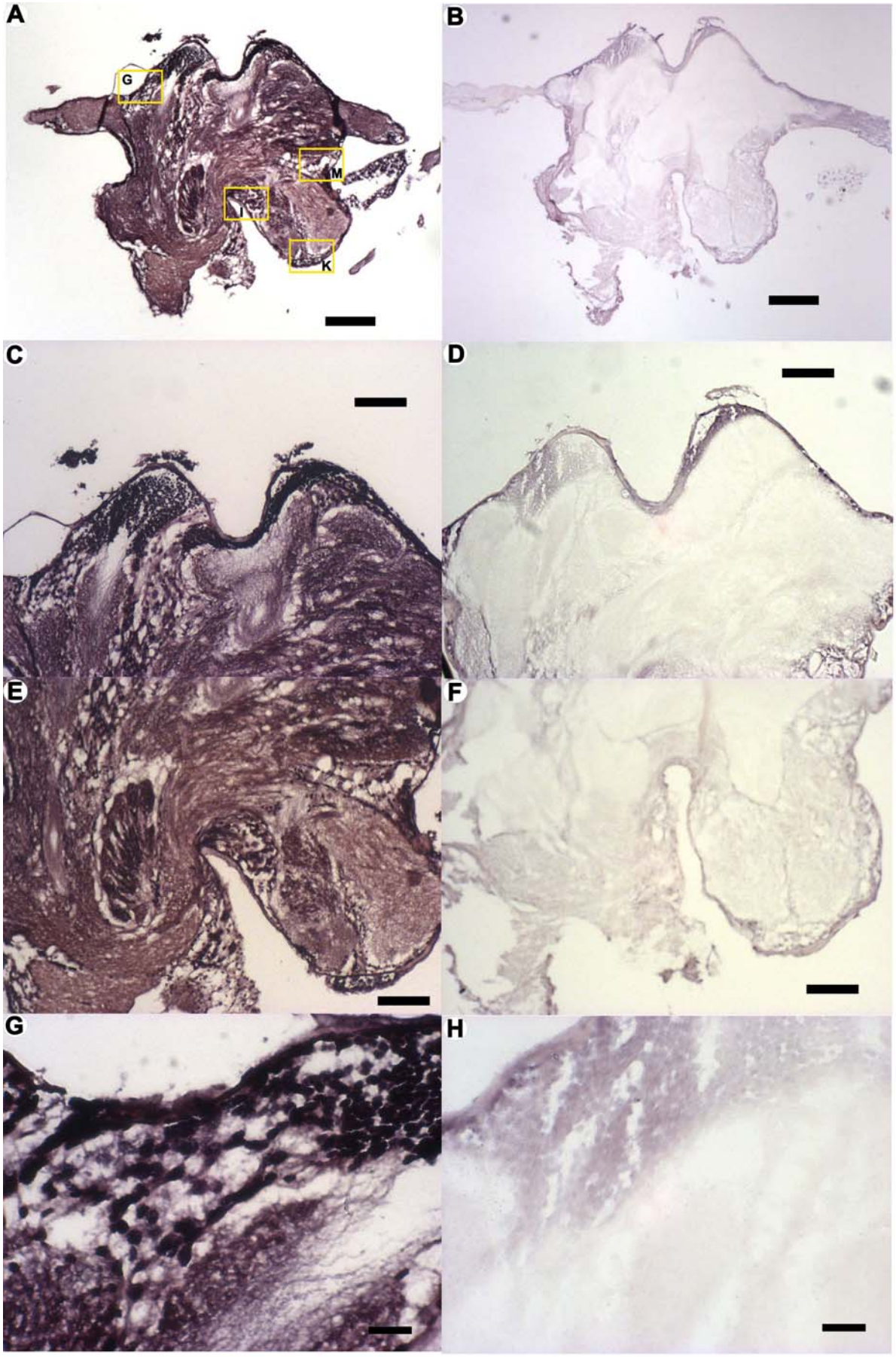
To detect nucleic acids in cells and tissues or specific genes in chromosomes, the technique of *in situ* hybridization was developed by Pardue and Gall (1969) and John et al. (1969). It allows the detection and the localization of specific nucleic acid sequences in cells and tissues since it preserves the morphology of the tissues, but is limited by its sensitivity (the nucleic acid copy number must be high enough). The polymerase chain reaction, which is a highly sensitive method for the detection of low amount of target nucleic acid sequences, has been extensively developed by morphologists in order to overcome the main limitation of *in situ* hybridization. A combination of PCR and *in situ* hybridization was at first described by Haase et al. (1990), although it was Tecott et al. (1988) who gave the idea with the description of the synthesis of cDNA in fixed tissue sections. This combination was termed *in situ* PCR (Ray et al., 1991). When *in situ* PCR was performed starting from an RNA template, the method was called RT *in situ* PCR (Nuovo, 1992), *in situ* cDNA (Chen and Fuggle, 1993) or *in situ* reverse transcriptase PCR (Komminoth et al., 1994) (for review see Morel et al., 1998; Nuovo, 2001). In general, the technique of *in situ* PCR or RT *in situ* PCR offered possible solutions to detect nucleic acids in cells and tissues by converting genetic information into visual signals that could be evaluated *in situ* while preserving cellular integrity and tissue morphology.

The RT *in situ* PCR technique was used for the localization of *Grybi*-AS B expression in various tissues of 1 day old adult virgin female crickets.

3.4.2.1. Localization of *Grybi*-AS B expression in the brain of 1 day old females

RT *in situ* PCR positive signals could be detected in the sections of the brain (Fig. 8). In the protocerebrum, positive signals were detected in the median neurosecretory cells, lateral neurosecretory cells and in nerve cells. The strongest signals were found in the region of the median neurosecretory cells, where more than one hundred cells were intensively stained. Inside the neurons only small granules could be detected. No signals were found in the sections of negative control. In the deuterocerebrum, RT *in situ* PCR positive signals could be detected in the lateral neurosecretory cells. In the tritocerebrum, strong positive signals were found in the neurosecretory cells. Again, no signal could be detected in the negative control sections of the deuterocerebrum and tritocerebrum. These results indicate that the *Grybi*-AS B gene is expressed in the medial neurosecretory cells as well as in lateral neurosecretory cells and neurons of the brain. The strongest expression was localized in the medial neurosecretory cells of the protocerebrum.

Fig. 8 Photomicrographs of *Grybi*-AS B expression localization by RT *in situ* PCR in sections of a 1 day old female brain. (A) Overview of the expression in the brain. Frames show the positions of corresponding pictures. (B) Matched control of the brain. (C) Expression in the protocerebrum, (D) Matched control. (E) Expression in the deuterocerebrum and tritocerebrum, (F) Matched control. (G) Higher magnification view of the expression in medial neurosecretory cells and lateral neurosecretory cells of the protocerebrum, (H) Matched control. (I) and (K) Higher magnification view of expression in neurosecretory cells of the tritocerebrum, (J) and (L) Matched controls. (M) Higher magnification view of the expression in the neurosecretory cells of the deuterocerebrum, (N) Matched control. (O) and (S) Expression in medial neurosecretory cells and in neurons of the protocerebrum, (P) and (T) Matched controls. (Q) and (U) Higher magnification views of expression in medial neurosecretory cells and neurons of the protocerebrum, (R) and (V) Matched controls. Scale bars in A, B=200 μm; in C, D, E, F, O, P, S, T=100 μm; in G, H, I, J, K, L, M, N, Q, R, U, V=20 μm.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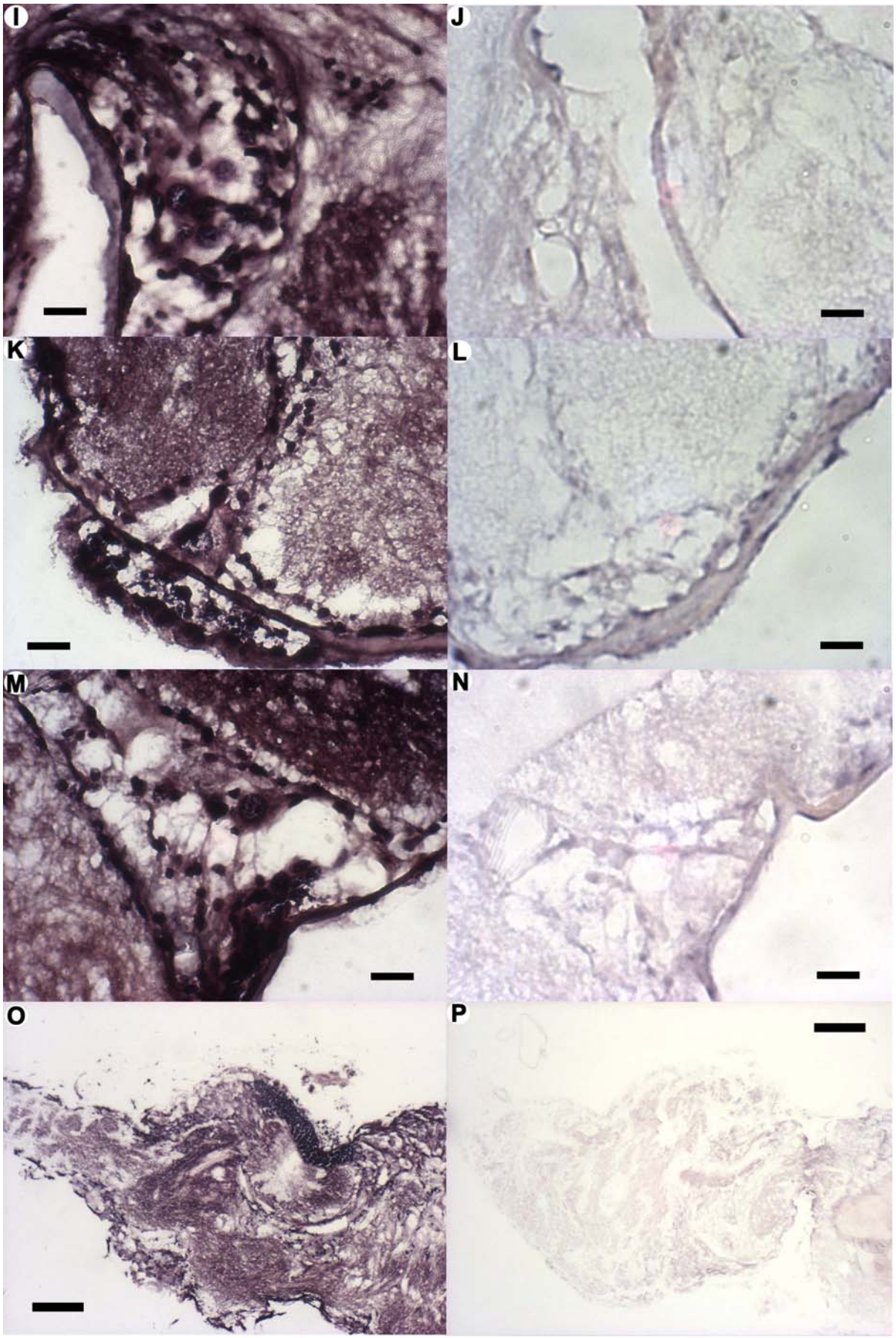
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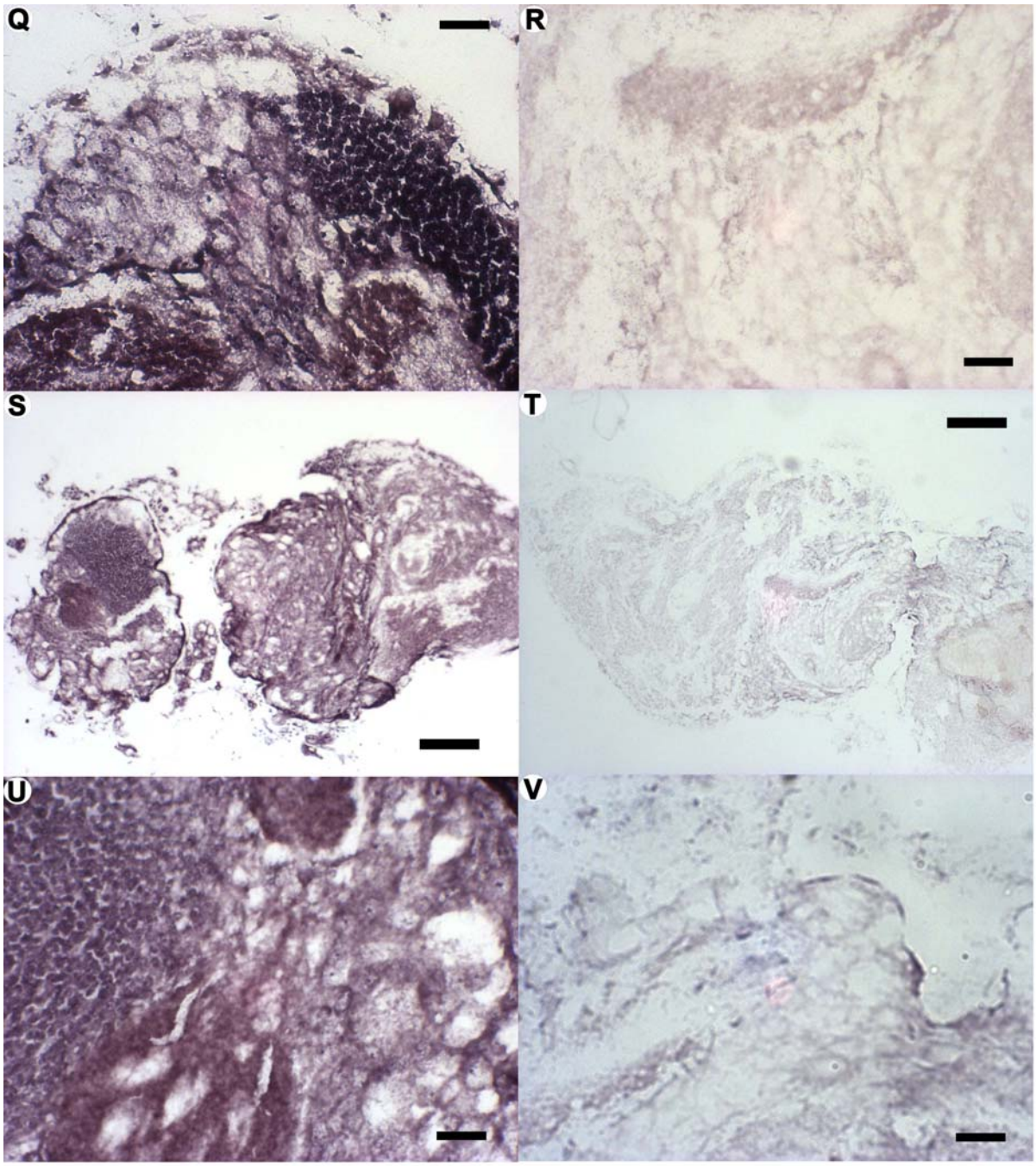
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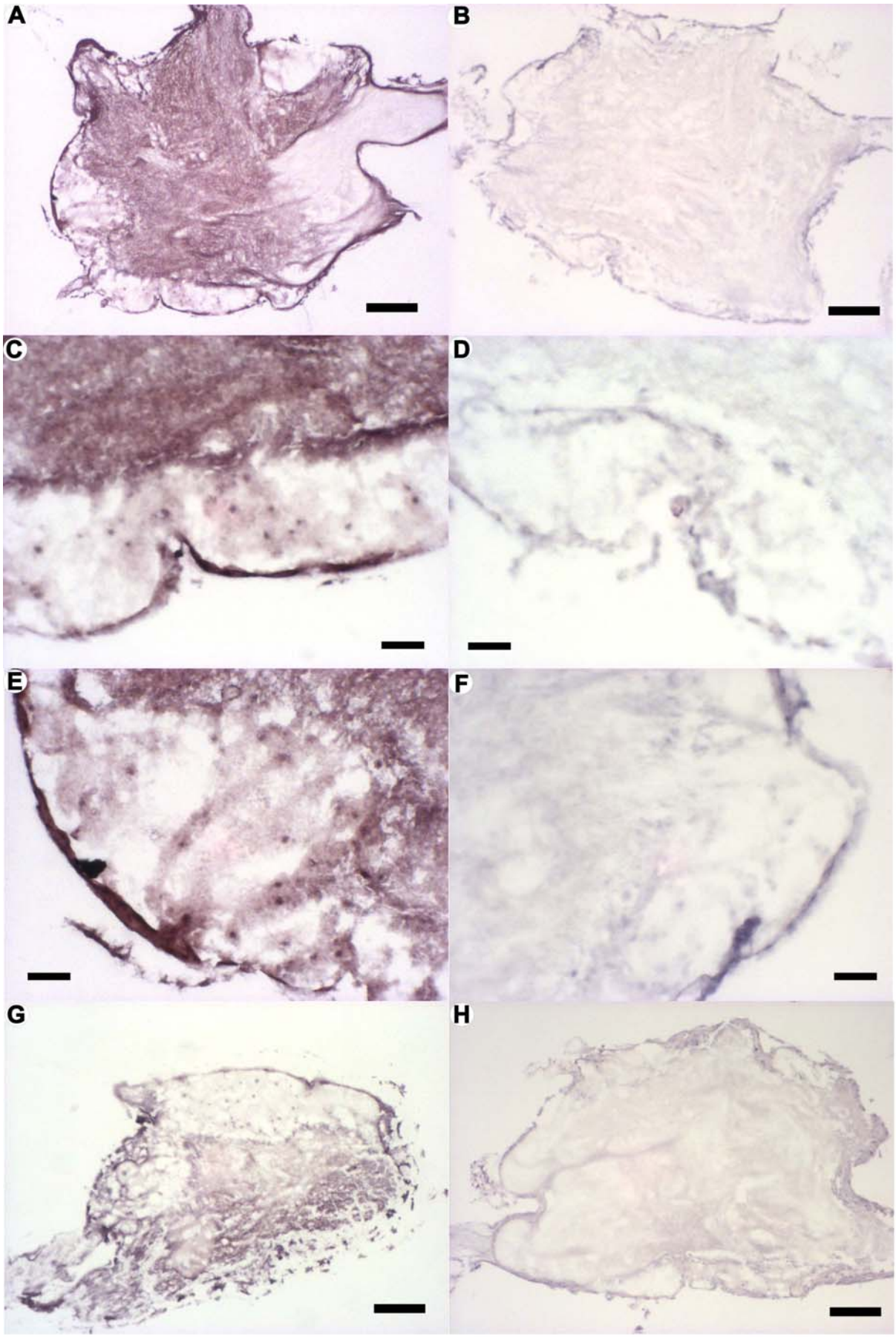
3.4.2.2. Localization of the *Grybi*-AS B expression in the suboesophageal ganglion of 1 day old females

RT *in situ* PCR positive signals could be detected in the sections of the suboesophageal ganglion (SOG). Positive signals were found in the neurosecretory cells which surround the medulla. In the negative control sections, no signals could be detected (Fig. 9). .

Fig. 9 Photomicrographs of *Grybi*-AS B expression by RT *in situ* PCR in sections of a 1 day old female suboesophageal ganglion. (A) and (G) Overviews of the expression in the suboesophageal ganglion, (B) and (H) Matched controls. (C), (E) and (I) Higher magnification views of the expression in neurosecretory cells of the suboesophageal ganglion, (D), (F) and (J) Matched controls. Scale bars in A, B, G, H=100 µm; in C, D, E, F, I, =20 µm.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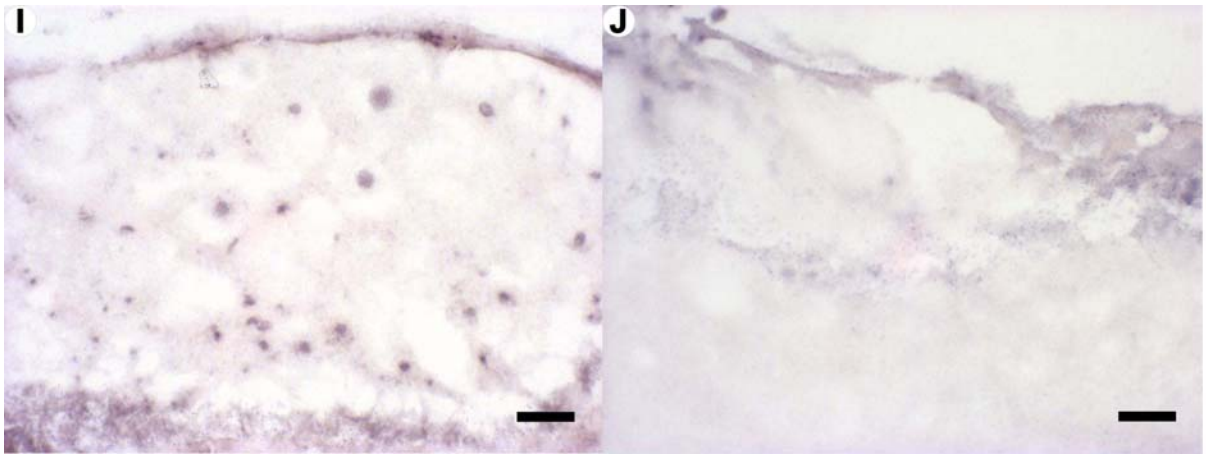
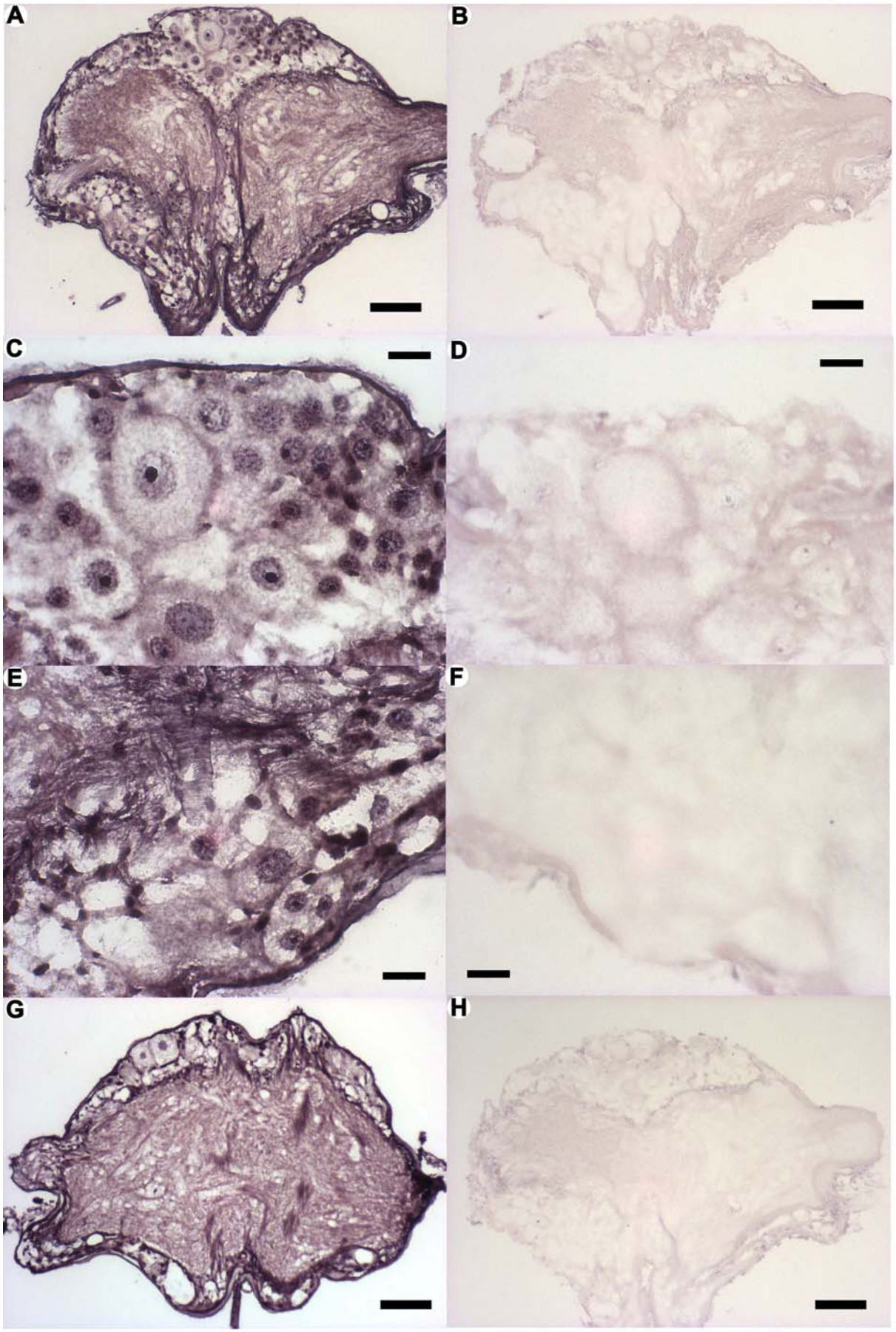


3.4.2.3. Localization of *Grybi*-AS B expression in the thoracic ganglion of 1 day old females

There are three thoracic ganglia in the cricket, which are nearly identical in shape and size. RT *in situ* PCR positive signals were visible in the sections of the thoracic ganglia. In the cortex of the thoracic ganglia, positive signals were detected not only in the cytoplasm of neurosecretory cells, but also in some nuclei of neurosecretory cells (Fig. 10). No signal was detected in the sections of negative controls .

Fig. 10 Photomicrographs of *Grybi*-AS B expression by RT *in situ* PCR in sections of a 1 day old female thoracic ganglion. (A) and (G) Overviews of the expression in the thoracic ganglia, (B) and (H) Matched controls. (C), (E), (I) and (K) Higher magnification views of the expression in neurosecretory cells of the thoracic ganglion, (D), (F), (J) and (L) Matched controls. Scale bars in A, B, G, H=100 μm; in C, D, E, F, I, J, K, L=20 μm.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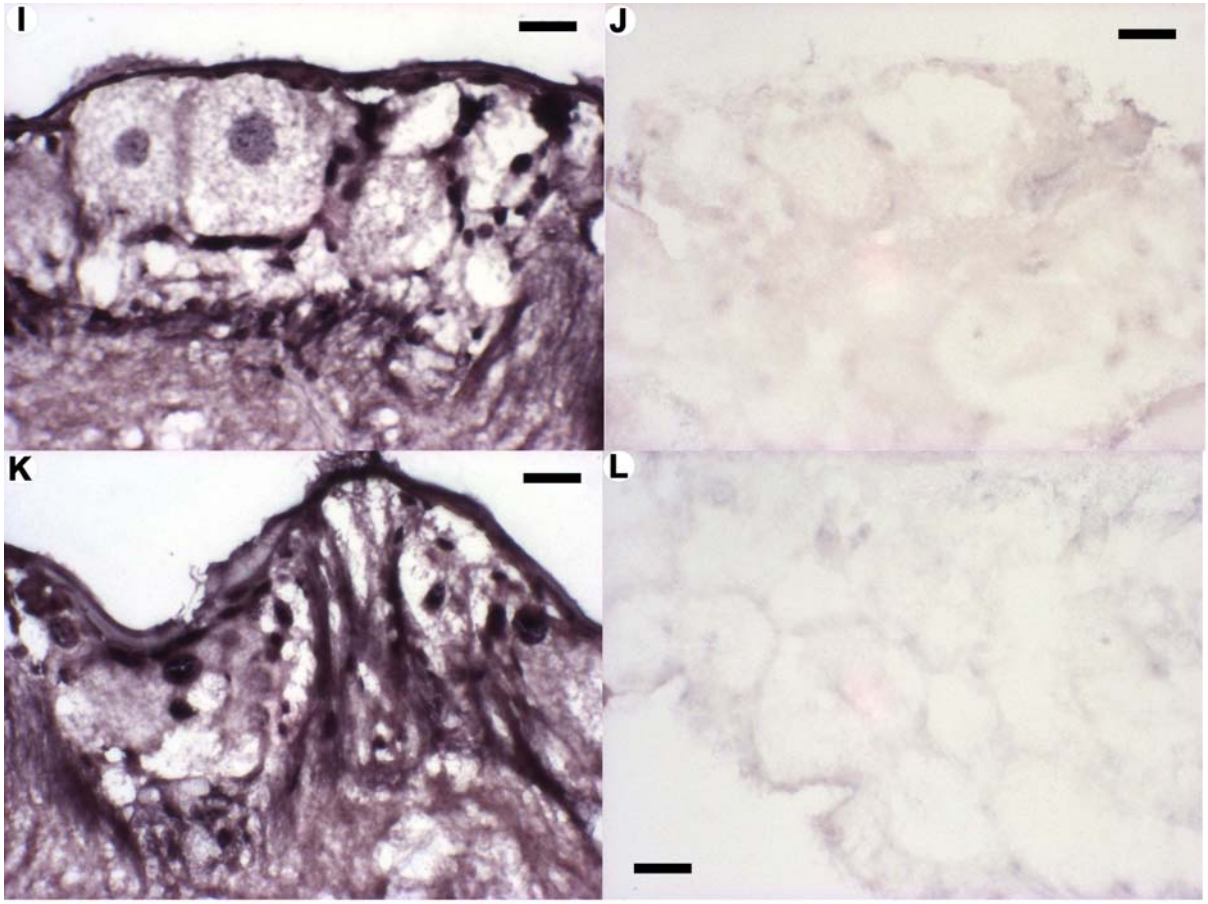
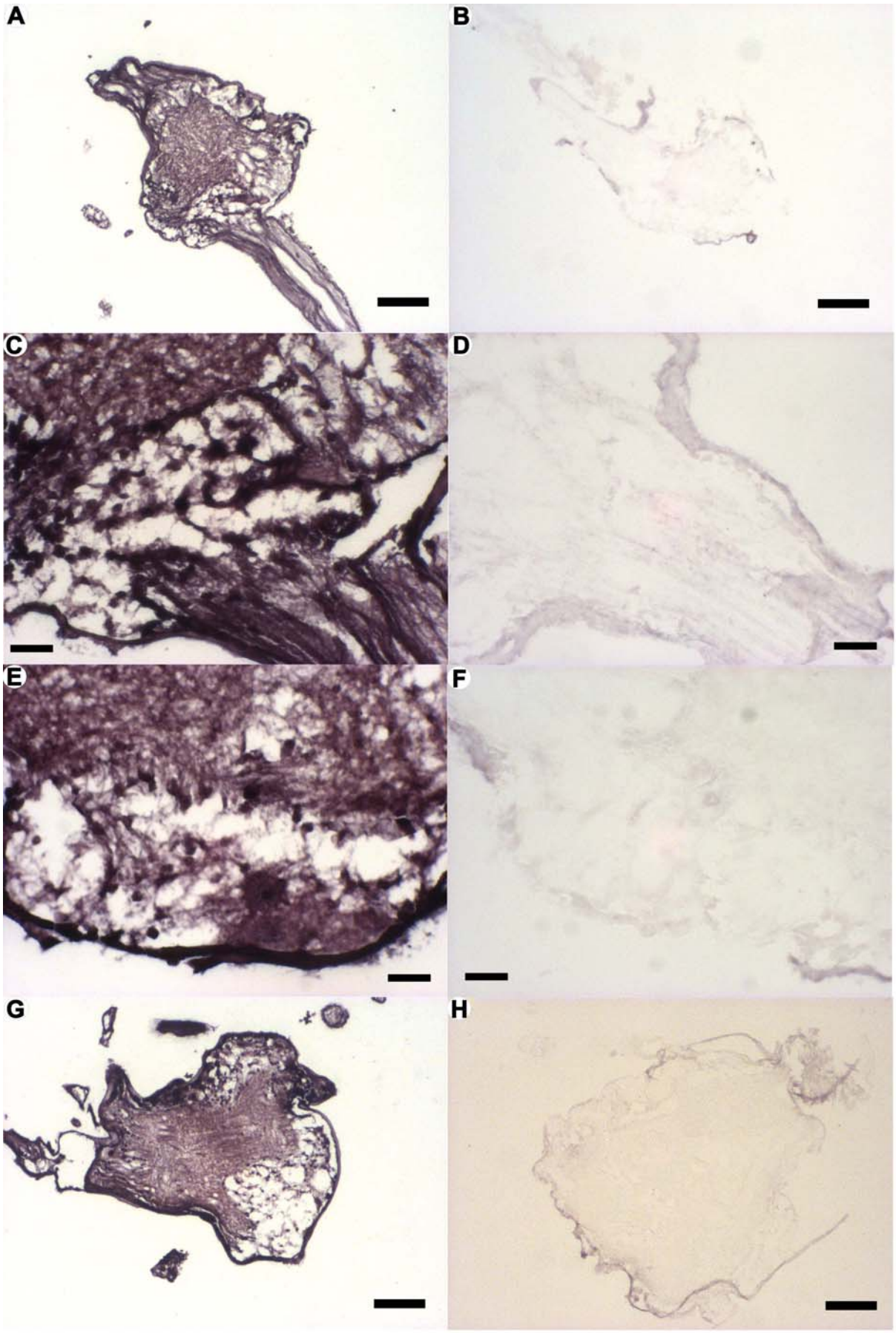


3.4.2.4. Localization of *Grybi*-AS B expression in the abdominal ganglia of 1 day old females

There are five abdominal ganglia in crickets. The first four frontal abdominal ganglia are nearly identical in size and shape, but smaller compared to the terminal abdominal ganglion. In the cortex of both kinds of abdominal ganglia, *in situ* RT-PCR positive signals were detected in the neurosecretory cells. No signal could be detected in the sections of negative controls (Fig. 11).

Fig. 11 Photomicrographs of *Grybi*-AS B expression by RT *in situ* PCR in sections of a 1 day old female abdominal ganglion. (A) Overview of the expression in the frontal abdominal ganglion, (B) Matched control. (C) and (E) Higher magnification views of the expression in neurosecretory cells of the frontal abdominal ganglia, (D) and (F) Matched controls. (G) Overview of the expression in the terminal abdominal ganglion, (H) Matched control. (I) and (K) Higher magnification views of the expression in neurosecretory cells of the terminal abdominal ganglion, (J) and (L) Matched controls. Scale bars in A, B, G, H=100 μm; in C, D, E, F, I, J, K, L=20 μm.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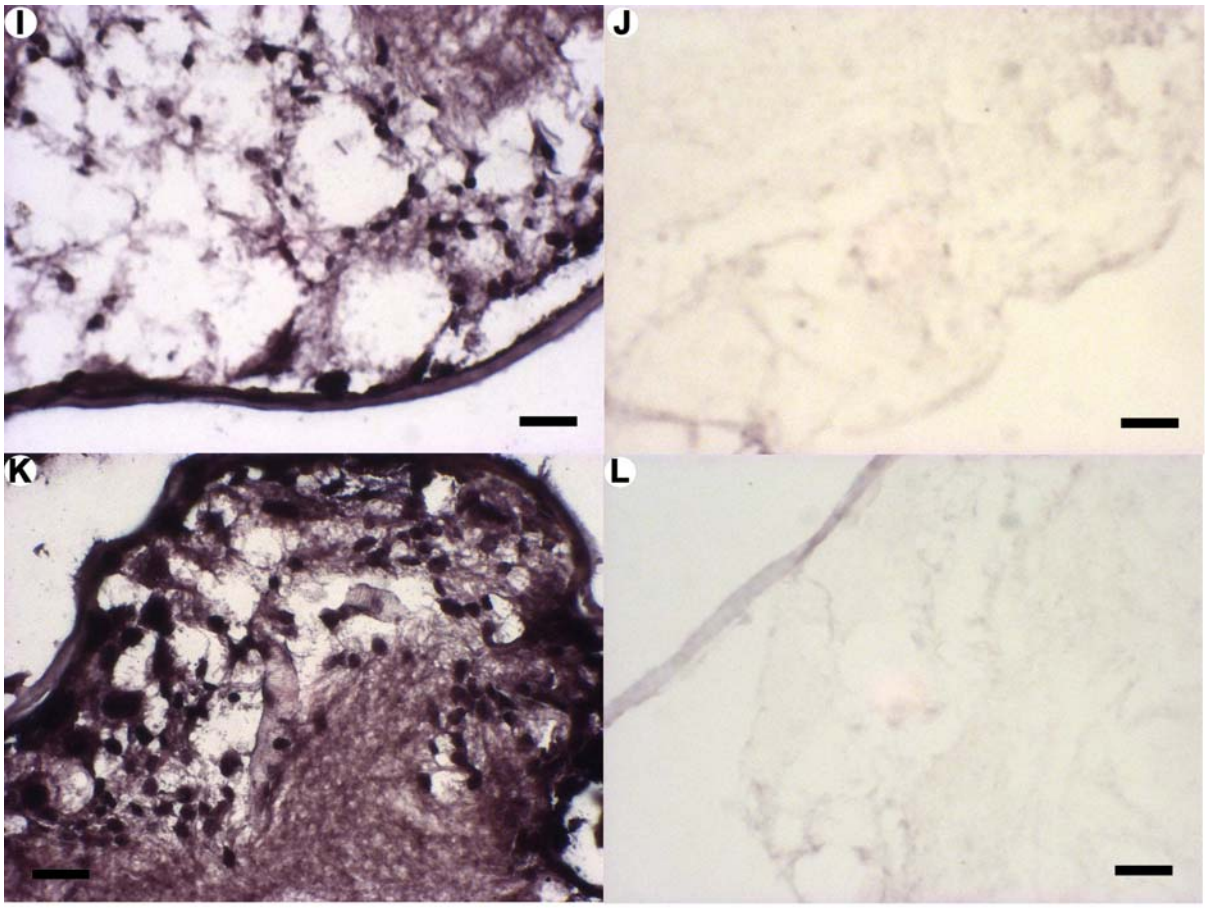


3.4.2.5. Localization of *Grybi*-AS B expression in the ovary of 1 day old females

The insect ovary may represent an important stimulatory or inhibiting regulator of CA activity (Stay et al., 1994b, 1996; Bylemans et al., 1998; Unnithan et al., 1998). Mature ovaries of *G. bimaculatus* contain various humoral factors which either stimulate or inhibit the biosynthesis of JH in the CA (Hoffmann et al., 1996). For these reasons, localization of *Grybi*-AS B gene expression in the ovary was investigated.

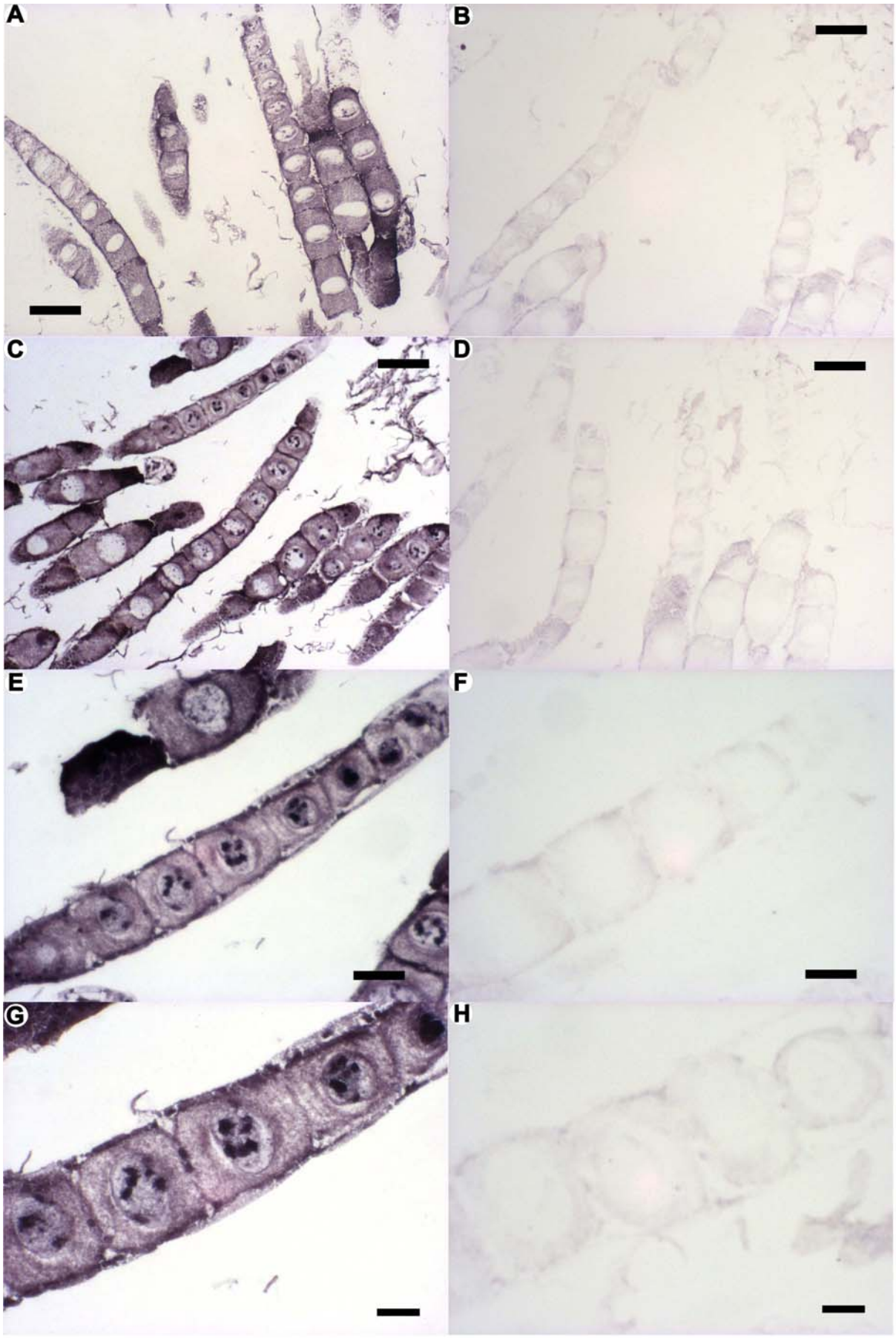
In the cricket ovary, which is of the panoistic type, each ovariole consists of a distal germarium in which oocytes are produced from oogonia, and a more proximal vitellarium in which yolk is deposited into the oocytes. These two regions reflect two phases of oocyte growth and development: the first is regulated directly by the oocyte´s genome and contains species-specific information. As the oocyte grows, its nucleus increases in size and is now known as the germinal vesicle. Transcription by the nuclear DNA appears to be suppressed soon after the beginning of yolk uptake. During yolk depostion, as the oocyte grows much more rapidly, the germinal vesicle is relatively smaller and, finally, the nuclear membrane

breaks down. The second phase of oocyte growth is mainly regulated by genes outside the oocyte, producing pools of molecules that will subsequently be involved in embryonic growth (Chapman, 1998).

3.4.2.5.1. Localization of *Grybi*-AS B expression in the germarium and primary oocytes

In the germarium, the RT *in situ* PCR positive signals were detected in the nuclei of primary oocytes. In the young primary oocytes near the terminal filament, condensed signals were detected in the nucleus, but no signals were detected in the prefollicular cells. With the ongoing development of oocytes, the signals were detected as separated granules and the intensity became weaker. At last, no signals could be detected in the nucleus (germinal vesicles) of vitellogenic oocytes, whereas the signals were detected in the follicular cells. The details of this procedure are shown in Fig. 12. No signal could be detected in the sections of negative controls.

Fig. 12 Photomicrographs of *Grybi*-AS B expression in the germarium of a 1 day old female ovary. (A) and (C) Overview of the expression in the germarium, (B) and (D) Matched controls. (E) Localization of the expression in primary oocytes during their development in the germarium, (F) Matched control. (G) and (I) Higher magnification views of the expression in primary oocytes of the germarium, (H) and (J) Matched controls. Scale bars in A-D=100 μm; E, F=40 μm; G-J=20 μm.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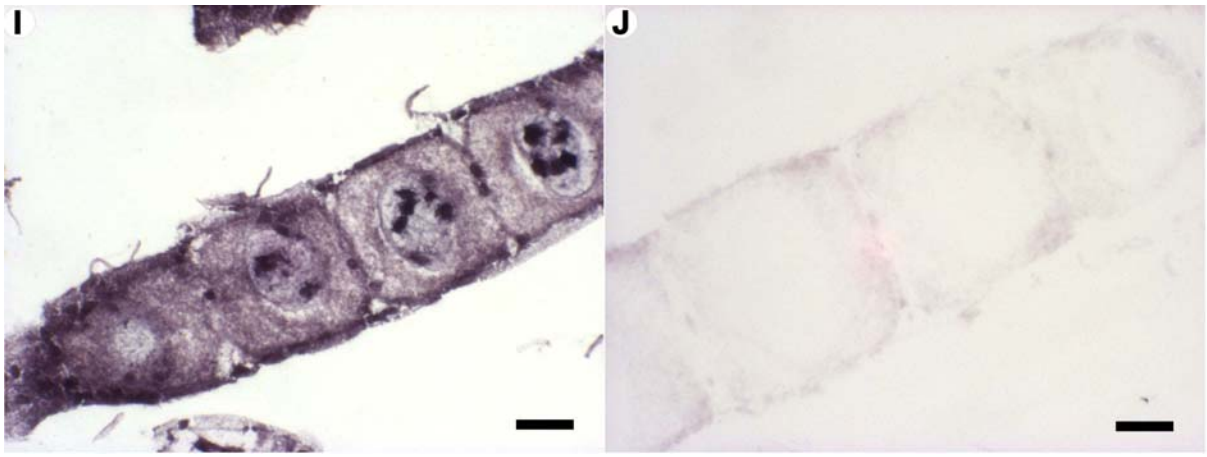
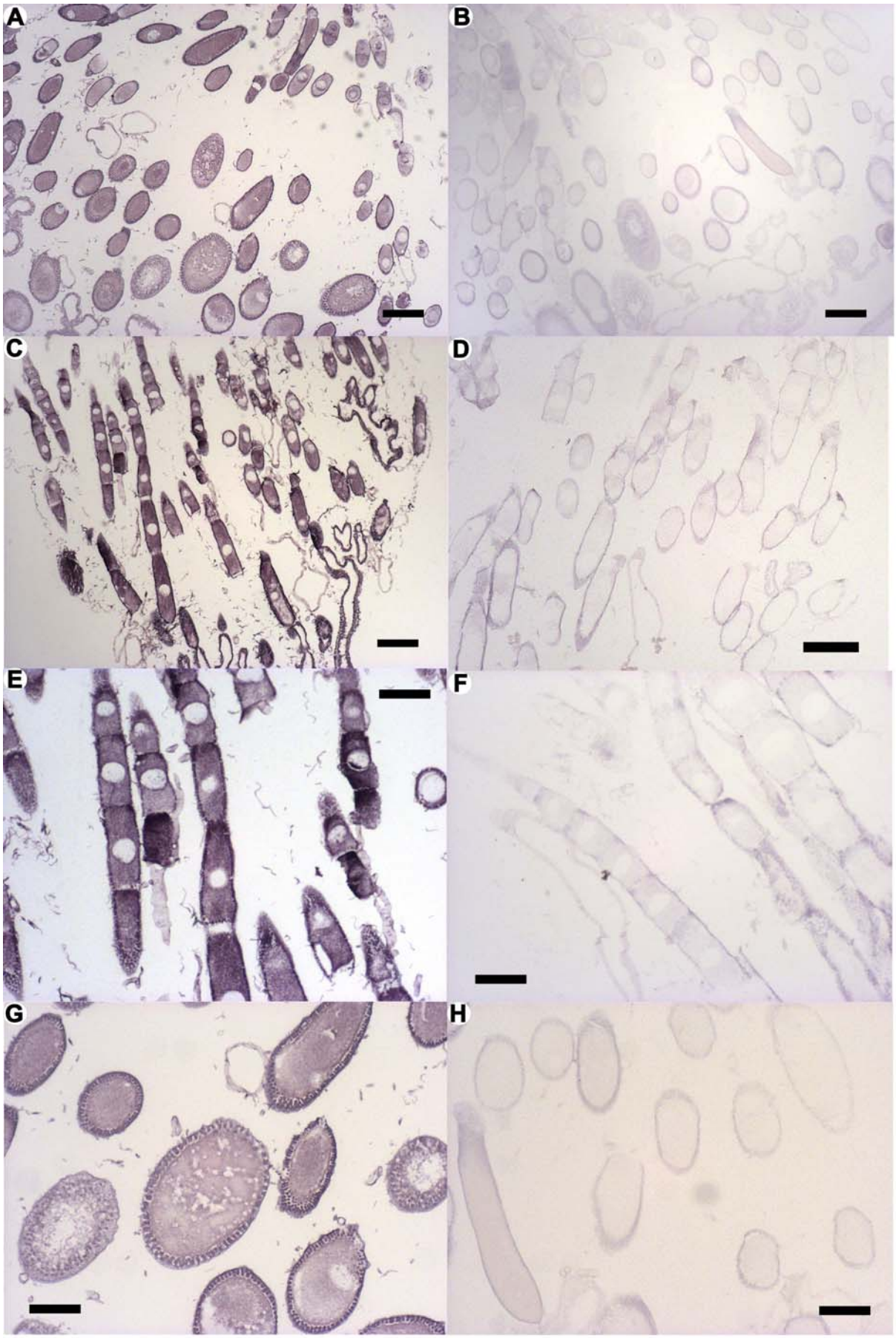


3.4.2.5.2. Localization of *Grybi*-AS B expression in the vitellarium

In the vitellarium, the oocytes were enveloped with follicular cells. The RT *in situ* PCR positive signals were detected in the follicular cells but no signals were detected in the germinal vesicles or in the cytoplasm of the oocytes. No signal could be detected in the sections of negative controls (Fig. 13).

Fig. 13 Photomicrographs of *Grybi*-AS B expression in the vitellarium of a 1 day old female ovary. (A) and (C) Overview of the expression in the vitellarium, (B) and (D) Matched controls. (E) and (G) Expression in the oocytes in the vitellarium, (F) and (H) Matched controls. (I), (K), (M), (O), (Q), (S) and (U) Higher magnification views of the expression in follicular cells enveloping the oocytes in the vitellarium, (J), (L), (N), (P), (R), (T) and (V) Matched controls. Scale bars in A-D=200 µM; E-H=100 µM; I-V=20 µM.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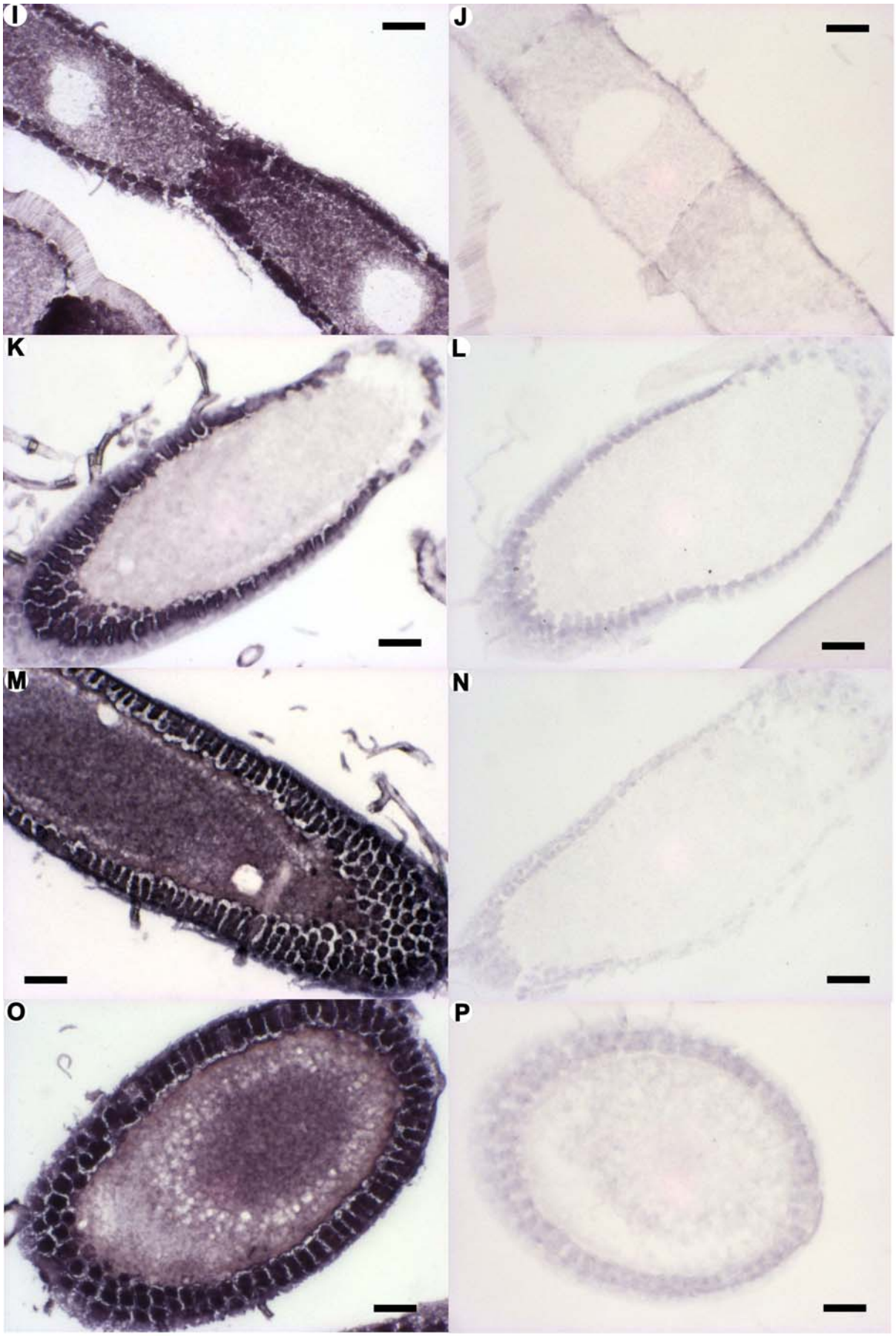
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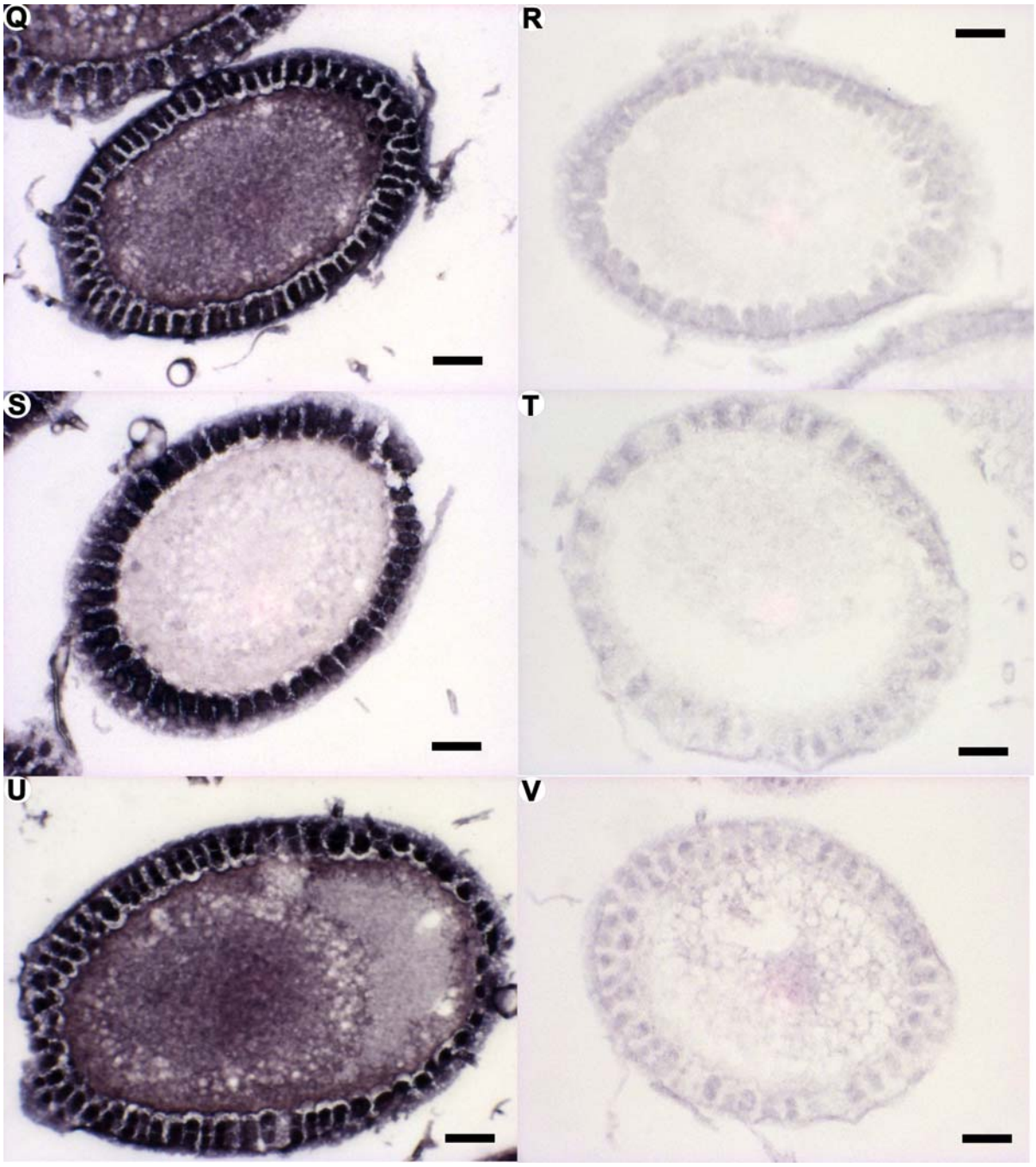
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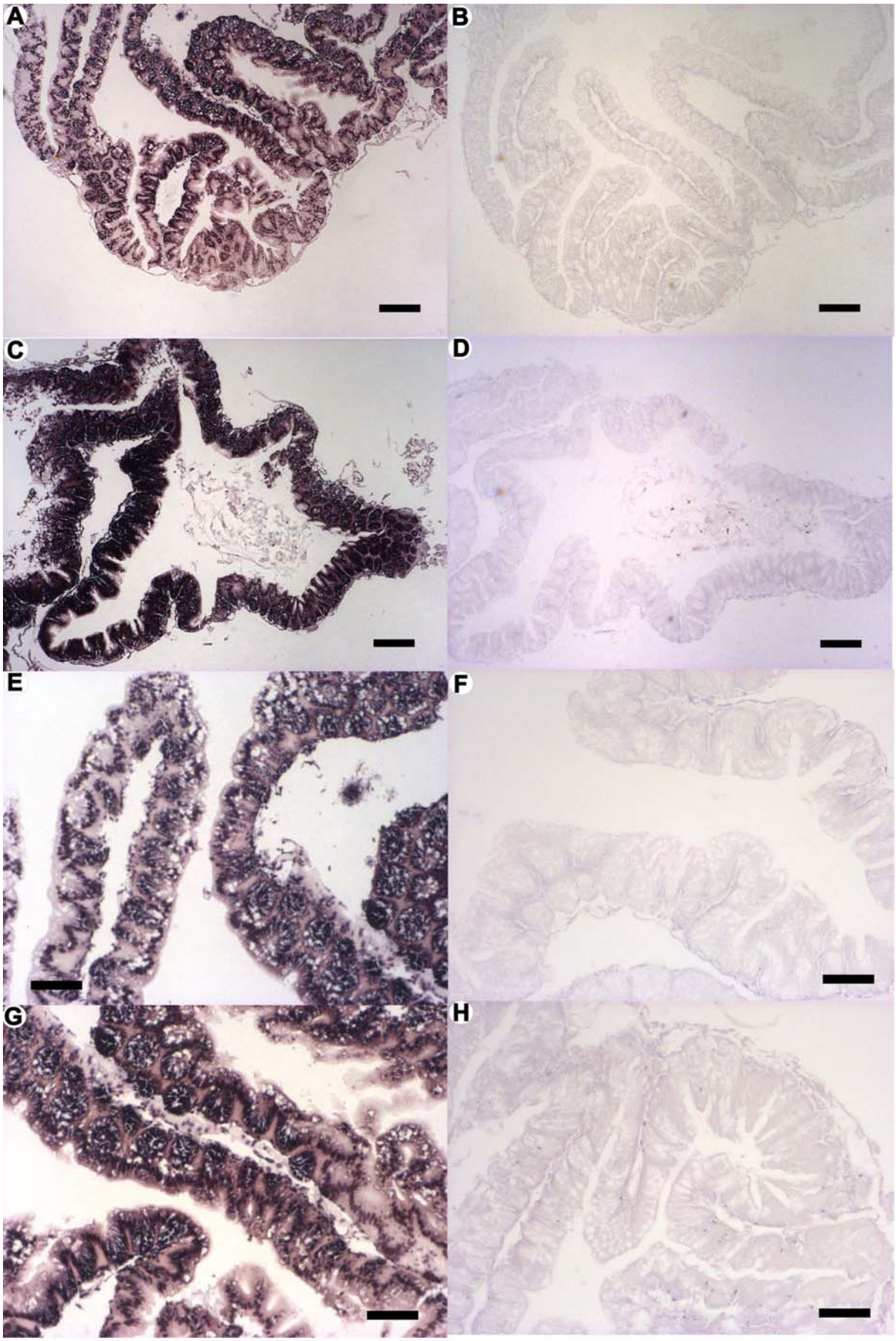
3.4.2.6. Localization of *Grybi*-AS B expression in the caecum and midgut of 1 day old females

Caecum and midgut of *G.bimaculatus* have similar anatomic structure and were used together for the studies on the localization of *Grybi*-AS B gene expression. In the sections of caecum and midgut, RT *in situ* PCR positive signals were detected in endocrine secretory and epithelial cells. No signals were detected in the sections of negative controls (Fig. 14).

Fig. 14 Photomicrographs of *Grybi*-AS B expression in sections of the caecum and midgut of 1 day old females. (A) Overview of the expression localization in the caecum, (B) Matched control. (C) Overview of the expression in the midgut, (D) Matched control. (E) and (G) Expression in endocrine secretory and epithelial cells in caecum and midgut, (F) and (H) Matched controls. (I), (K), (M) and (O) Higher magnification views of the expression in endocrine secretory and epithelial cells of the caecum and midgut. (J), (L), (N) and (P), Matched controls. Scale bars in A-D=200 μm; E-H=100 μm; I-P=20 μm.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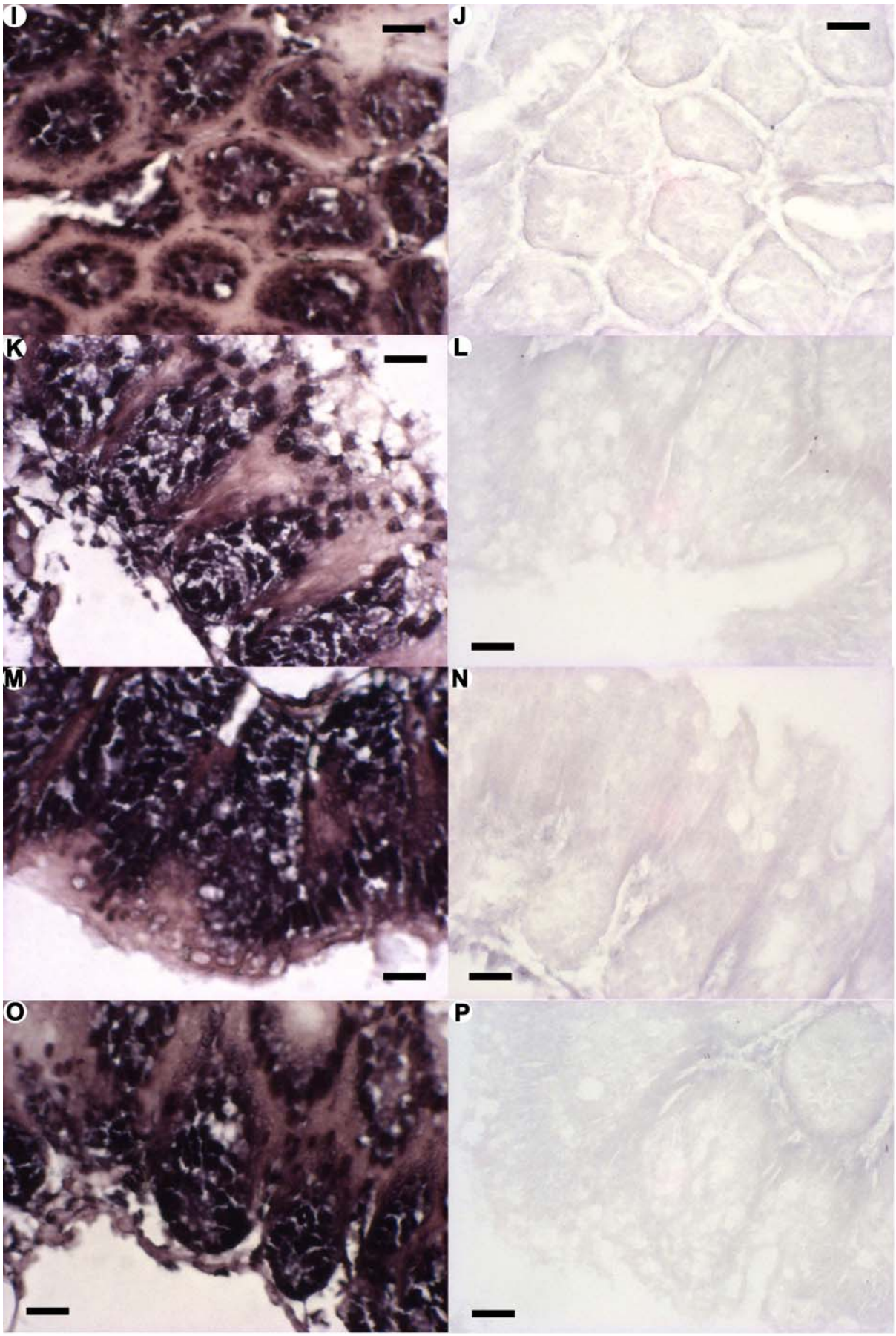
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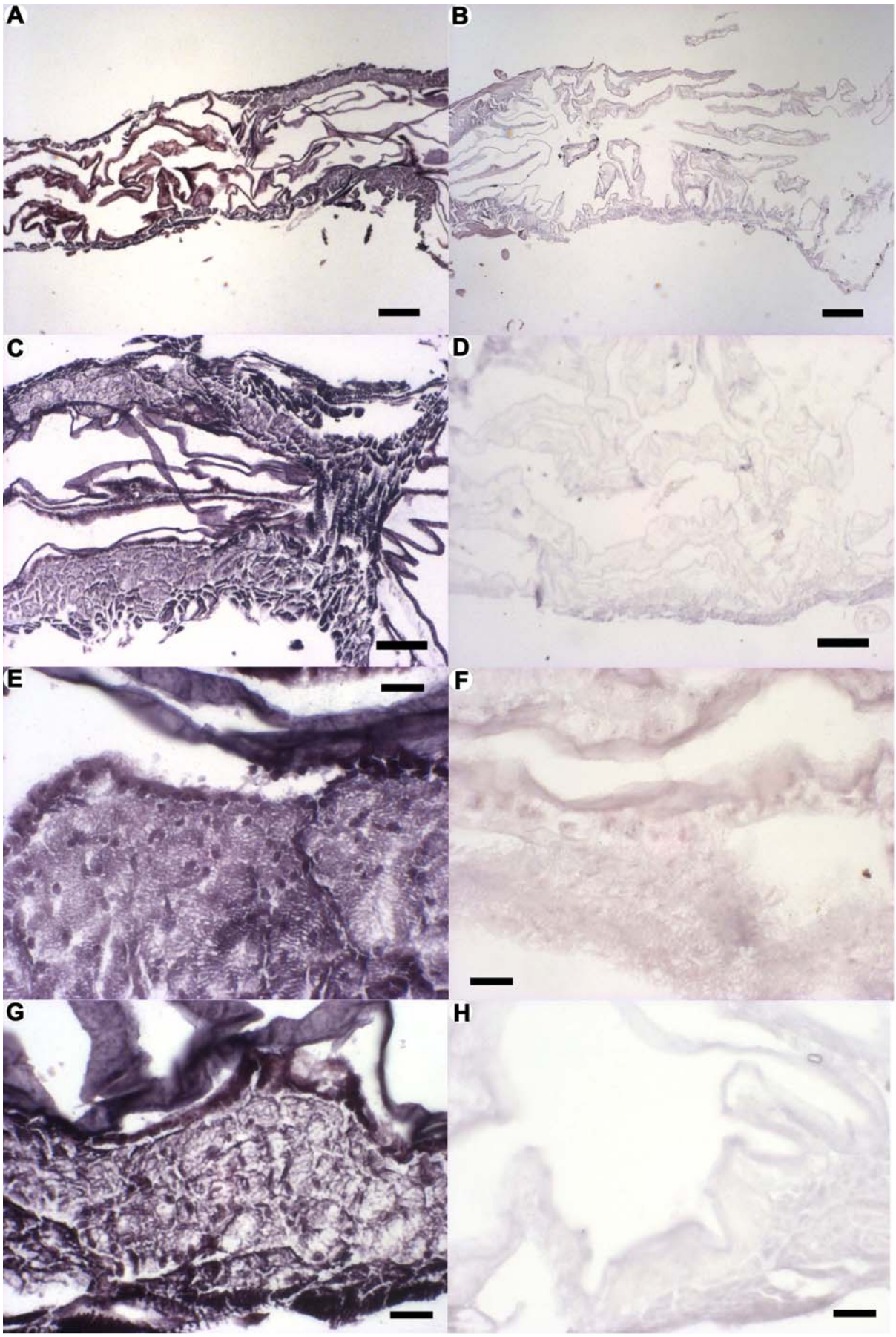
3.4.2.7. Localization of *Grybi*-AS B expression in the hindgut of 1 day old females

RT *in situ* PCR positive signals could be detected in the sections of the hindgut. Positive signals were detected in the gut epithelium cells and in muscles. In the cuticle, no signal was detected, except some unspecific absorption of colour-substrate. No signal was detected in the sections of negative controls (Fig. 15).

Fig. 15 Photomicrographs of *Grybi*-AS B expression in sections of a 1 day old female hindgut. (A) Overview of the expression in the hindgut, (B) Matched control. (C) Expression in epithelium cells and muscles of the hindgut, (D) Matched control. (E) and (G) Higher magnification views of the expression in epithelium cells and muscles of the hindgut, (F) and (H) Matched controls. Scale bars in A, B=200 μm; C, D=100 μm; E-H=20 μm.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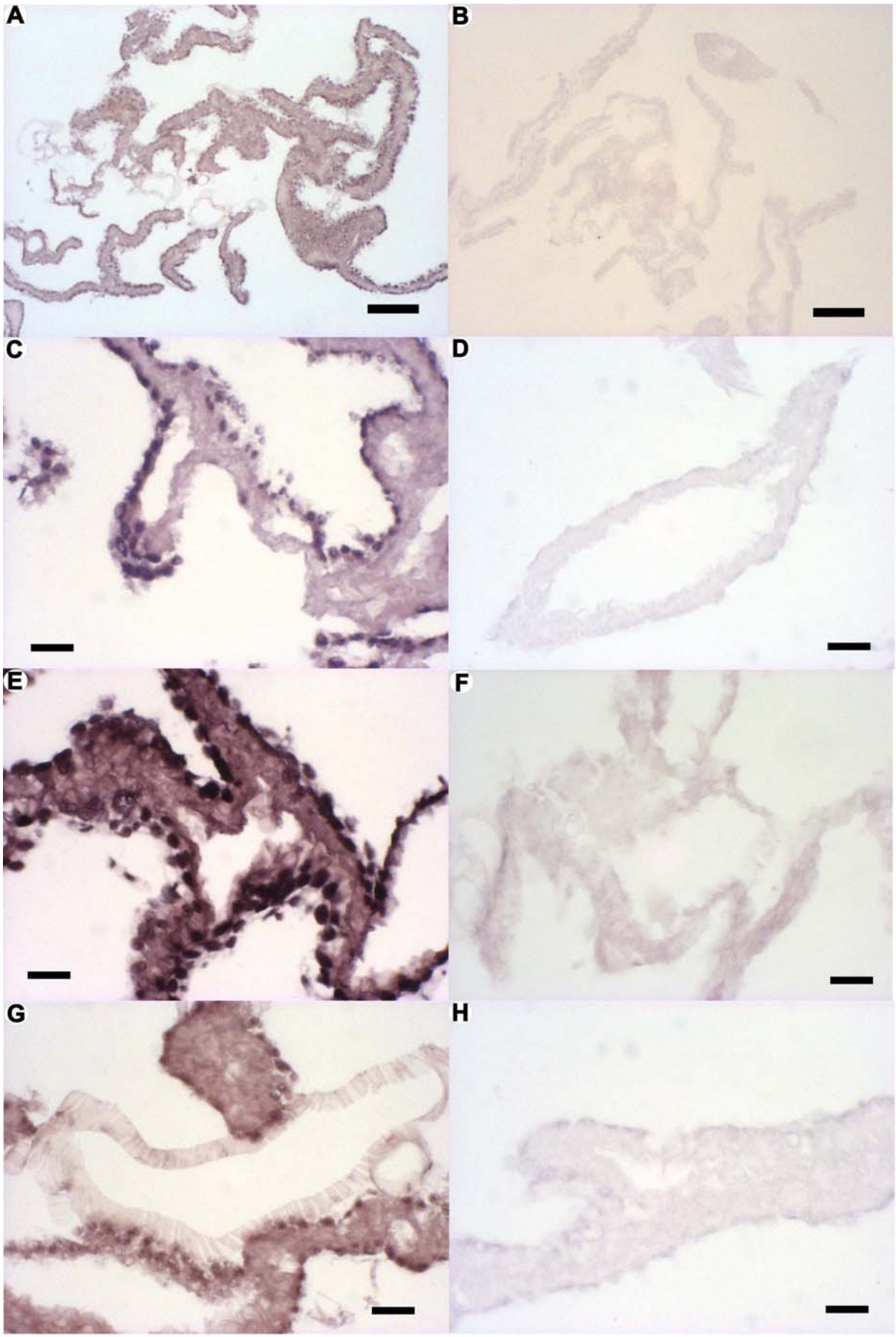
3.4.2.8. Localization of *Grybi*-AS B expression in the accessory reproductive glands of 1 day old females

RT *in situ* PCR positive signals could be detected in the sections of the accessory reproductive glands. Positive signals were detected in the epithelium cells of accessory reproductive glands. No signals were detected in the sections of negative controls (Fig. 16).

Fig. 16 Photomicrographs of *Grybi*-AS B expression in sections of 1 day old female accessory reproductive glands. (A) Overwiew of the expression in accessory reproductive glands, (B) Matched control. (C), (E) and (G) Higher magnification views of the expression in epithelium cells of the accessory reproductive glands, (D), (F) and (H) Matched controls. Scale bars in A, B=100 μm; C-H=20 μm.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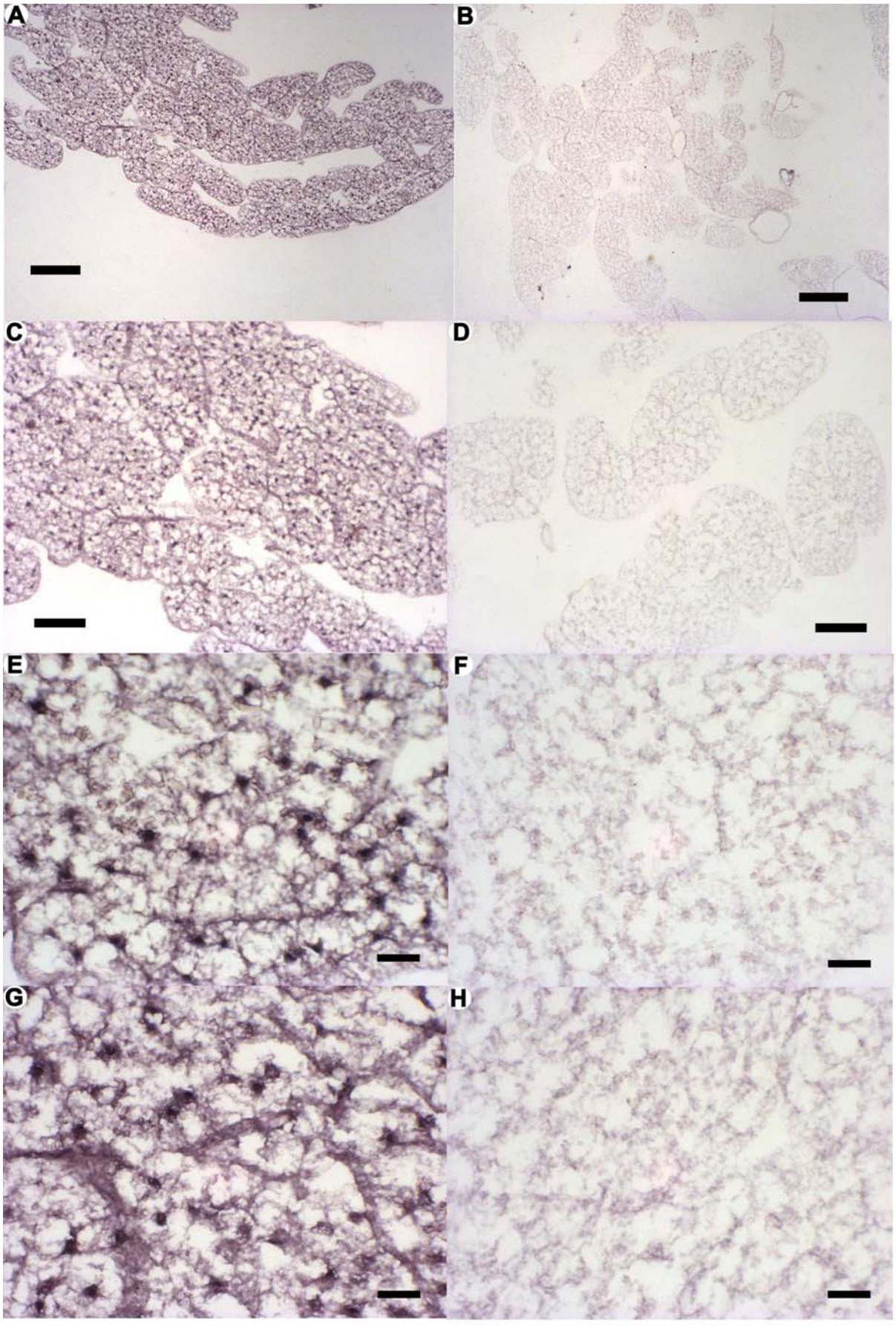
3.4.2.9. Localization of *Grybi*-AS B expression in the fat body of 1 day old females

Insect fat body is mainly for energy stores. Essential metabolic enzymes are found in the fat body of all insects along with the reserves which may be used for energy-demanding events such as flight, oogenesis and overwintering. RT *in situ* PCR positive signals could be detected in the sections of the fat body. No signal was detected in the sections of negative controls (Fig. 17).

Fig. 17 Photomicrographs of *Grybi*-AS B expression in sections of a 1 day old female fat body. (A) Overview of the expression in the fat body, (B) Matched control. (C) Expression in the fat body cells, (D) Matched control. (E) and (G) Higher magnification views of the expression in the fat body, (F) and (H) Matched controls. Scale bars in A, B=200 µm; C, D=100 µm; E-H=20 µm.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3.4.2.10. Localization of *Grybi*-AS B expression in thoracic muscles of 1 day old females

RT *in situ* PCR positive signals were detected in the sections of thoracic muscles. No signal was detected in the sections of negative controls (Fig. 18).

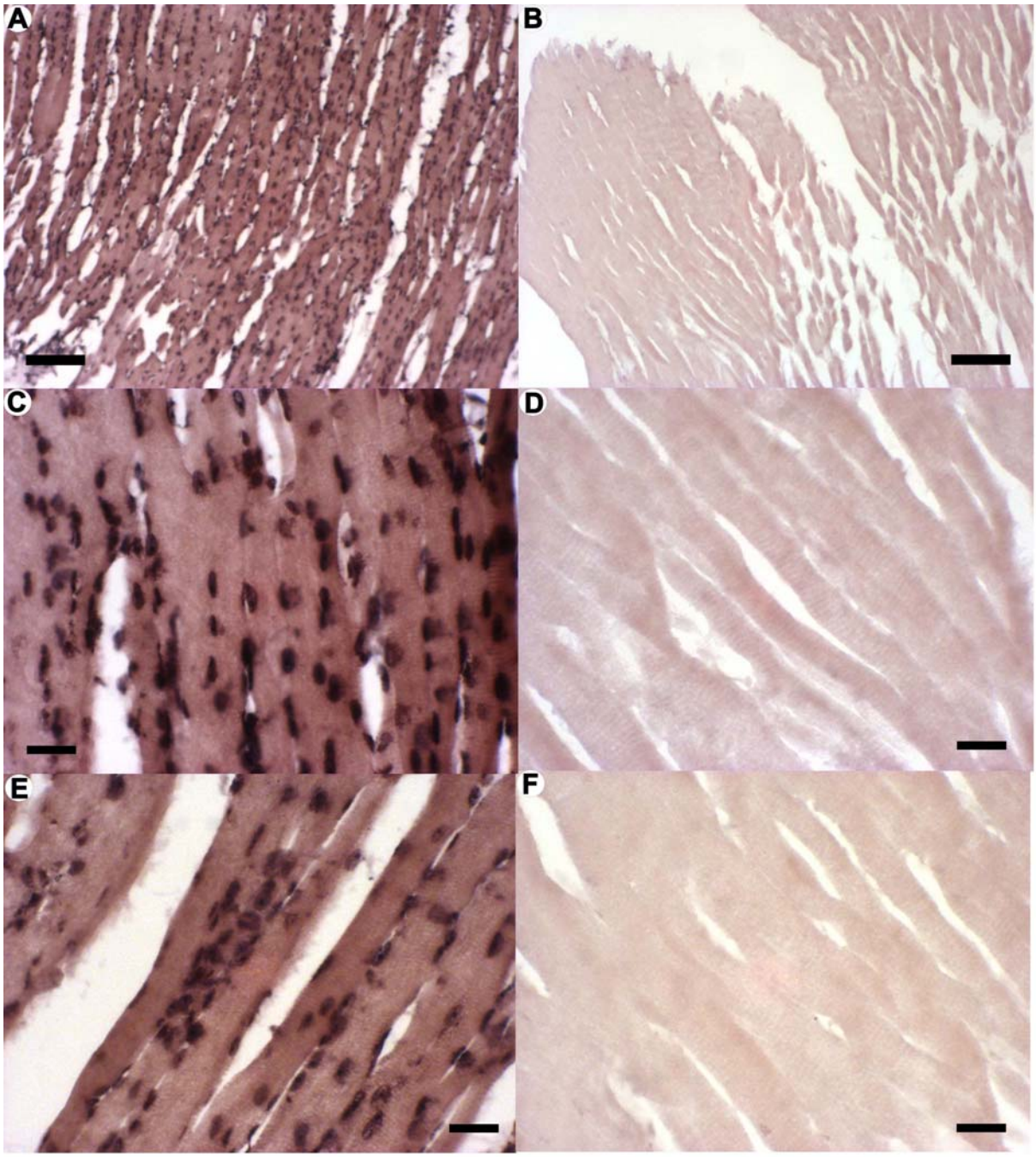

Fig. 18 Photomicrographs of *Grybi*-AS B expression sections of 1 day old female thoracic muscles. (A) Expression in the thoracic muscles, (B) Matched control. (C) and (E) Higher magnification views of the expression in thoracic muscles, (D) and (F) Matched controls. Scale bars in A, B=100 µm; C-F=20 µm.

3.4.2.11. Examination of the diffusion of the RT *in situ* PCR products

For each RT *in situ* PCR experiment, an aliquot of master mixture was used for liquid RT-PCR using mRNA as template individually. To detect whether the RT *in situ* PCR products have diffused from the site of amplification, supernatants of the amplification mixture were removed from the top of the slides of the test and the negative control randomly, then 10 µl of each supernatant was submitted to agarose gel electrophoresis. After gel electrophoresis, liquid RT-PCR using mRNA as template yielded the expected band. In contrast, no visible band could be detected from the supernatants of the RT *in situ* PCR amplification mixture (Fig. 19). These results indicate that the RT *in situ* PCR products were amplified inside the cells/tissues.

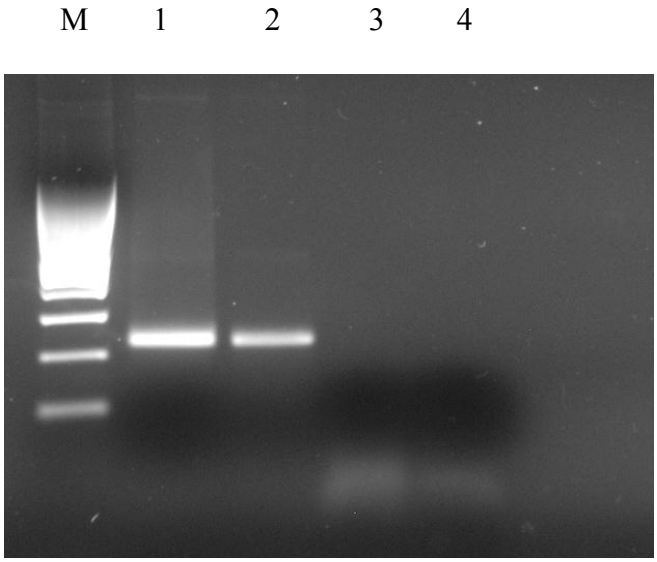

Fig. 19 Examination of the diffusion of the RT *in situ* PCR products by agarose gel electrophoresis stained with ethidiumbromide. A 100 bp marker was run and size marked on the left. Lane (1) Liquid RT-PCR product amplified with one-step RT-PCR kit. Lane (2) Liquid RT-PCR product amplified with *Tth* DNA polymerase. Lane (3) Supernatant of the RT *in situ* PCR amplification mixture from a test slide. Lane (4) Supernatant of the RT *in situ* PCR amplification mixture from a negative control slide.

3.4.3. Age-dependency of *Grybi*-AS B gene expression in different tissues

Allatostatin B type expression levels in various tissues of adult virgin and mated female and male crickets, respectively, of different ages were elucidated by RNA dot blot hybridization. This method was originally performed by spotting a small sample of the RNA preparation onto dry nitrocellulose, which was then dried, hybridized with a specific ^{32}P-labelled probe, and exposed to X-ray film (Kafatos et al., 1979). Because of the large and variable size of the spots, making accurate quantification unfeasible, this technique was improved by designing the filtration manifolds to accept a large number of samples and to deposit the nucleic acids onto the nitrocellulose in a fixed pattern that allows the results to be quantified by scanning densitometry (Brown et al., 1983; Chapman et al., 1983). Compared with gel electrophoresis methods, this method allowed the rapid analysis of numerous small samples for the sequence of interest and was less time consuming.

3.4.3.1. *Grybi*-AS B expression in the brain

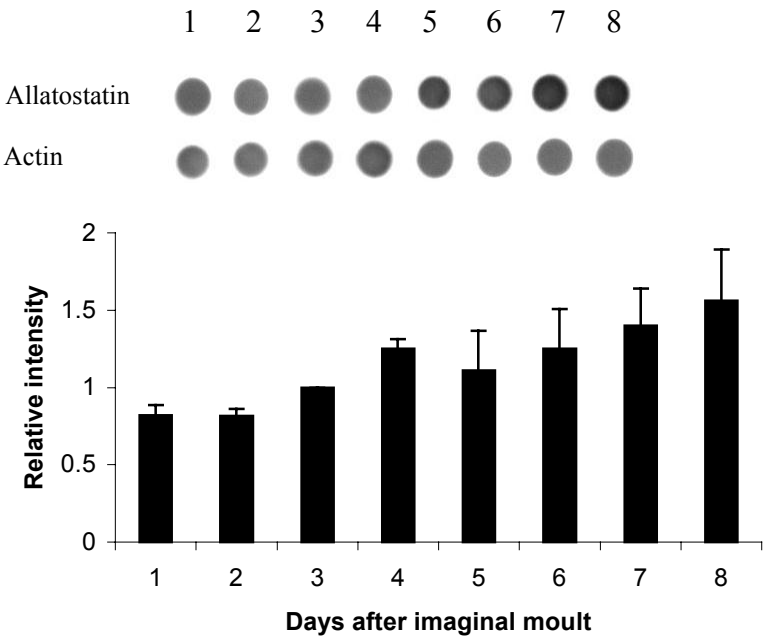

Fig. 20 RNA dot blot analysis of the allatostatin B type expression in the brain of adult virgin female crickets *G. bimaculatus*. Hybridization has been done with the allatostatin B and the actin (control) digoxigenin labelled RNA pobes. On the upper part of the figure the results of the dot blot are shown. The lower graph shows the analysis of the relative intensity of the dots by quantification of the integrated optical density, normalized to the actin probe. The values were calculated relative to that of 3 d-old crickets, which was set to 1. Mean values ± SD, n = 4.

Allatostatin B type expression in the brain of virgin females began to increase on day 3 after imaginal moult and increased smoothly until day 8 (Fig. 20).

In the brain of males, a peak value appeared earlier, on day 2 after imaginal moult (Fig. 21).

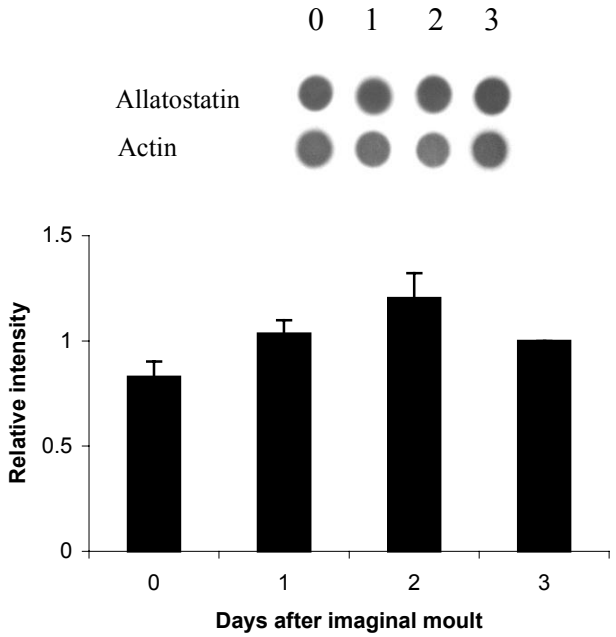

Fig. 21 RNA dot blot analysis of the allatostatin B type expression in the brains of adult male crickets. Details are given in Fig. 20. Mean values ± SD, n = 4.

3.4.3.2. *Grybi*-AS B expression in the ovary

After ecdysis, allatostatin type B expression in the ovary of virgin females decreased from day 0 to day 2, but increasinged thereafter and reached a peak value on day 4. Then expression decreased again (Fig. 22).

In the ovary of mated females, the temporal expression pattern of allatostatin type B was different to that in virgin females. Expression decreased after moulting and remained at a low level from day 3 to day 7, the time period of intense egg production and oviposition in mated females (Fig. 23).

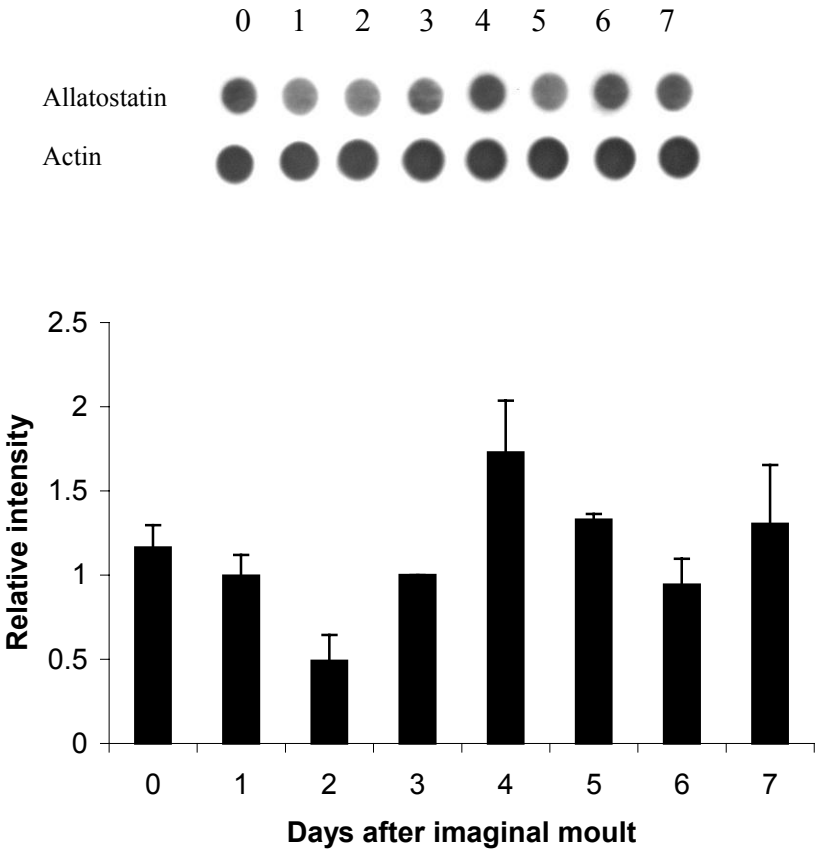

Fig. 22 RNA dot blot analysis of the allatostatin type B expression in the ovary of adult virgin female crickets. Details are given in Fig. 20. Mean values ± SD, n = 4.

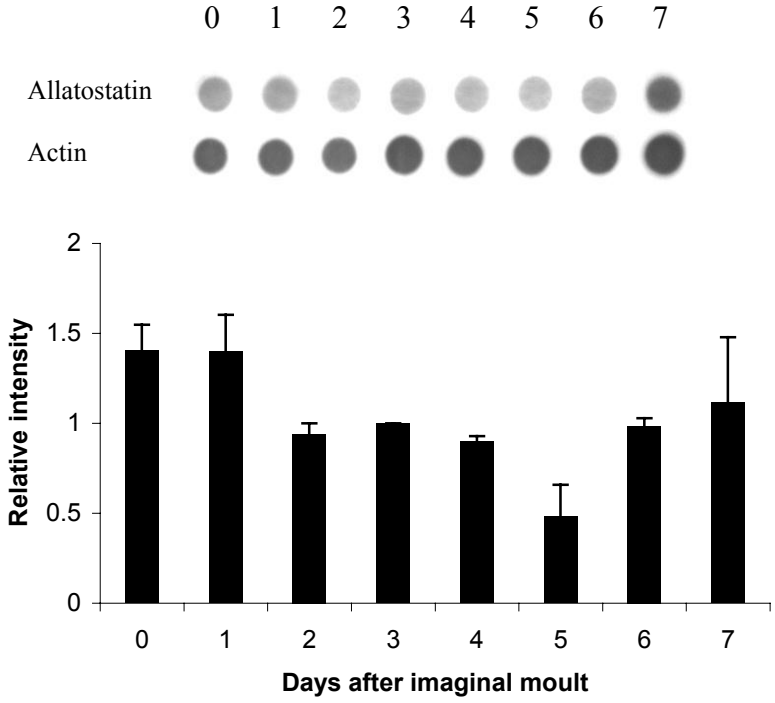

Fig. 23 RNA dot blot analysis of the allatostatin type B expression in the ovary of adult mated female crickets. Details are given in Fig. 20. Mean values ± SD, n = 4.

3.4.3.3. *Grybi*-AS B expression in the caecum and midgut of virgin females

Caecum and midgut of virgin females were used together for allatostatin B type expression studies. From day 1 to day 5 after imaginal moult, allatostatin B type expression was generally low. It then increased on day 6, remained at a high level on day 7, and then drastically decreased to day 8 (Fig. 24).

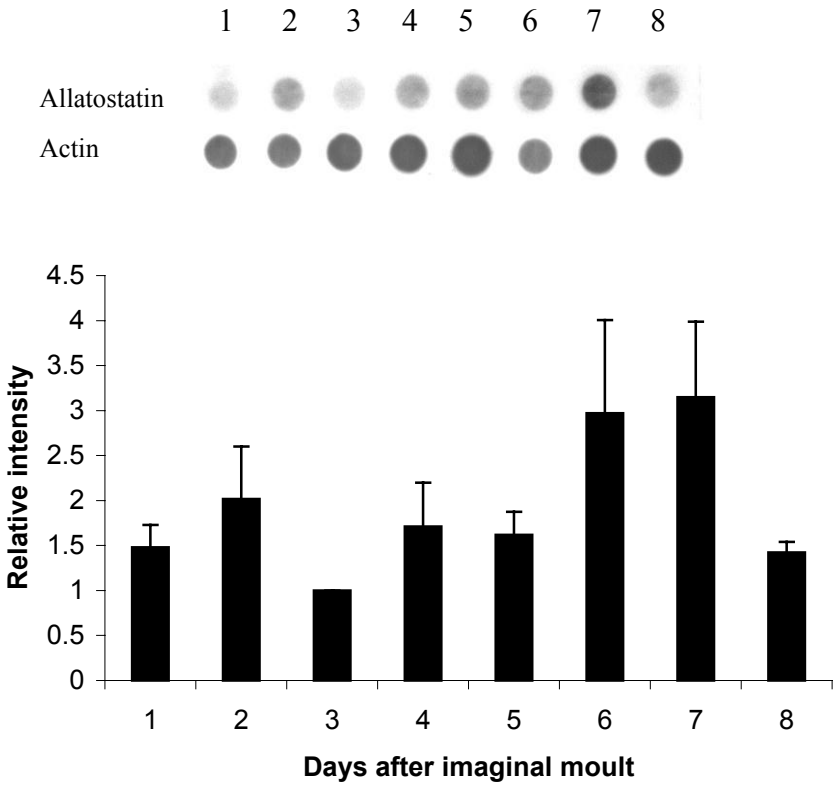

Fig. 24 RNA dot blot analysis of the allatostatin type B expression in caecum and midgut of adult virgin female crickets. Details are given in Fig. 20. Mean values ± SD, n = 4.

3.4.3.4. *Grybi*-AS B expression in the hindgut of virgin females

Allatostatin type B expression in the hindgut of virgin females was high after ecdysis, but decreased thereafter (days 1 and 2). Another high value of expression was observed on day 3, followed by a decrease on day 4. The expression level was higher again from day 5 to day 7 (Fig. 25).

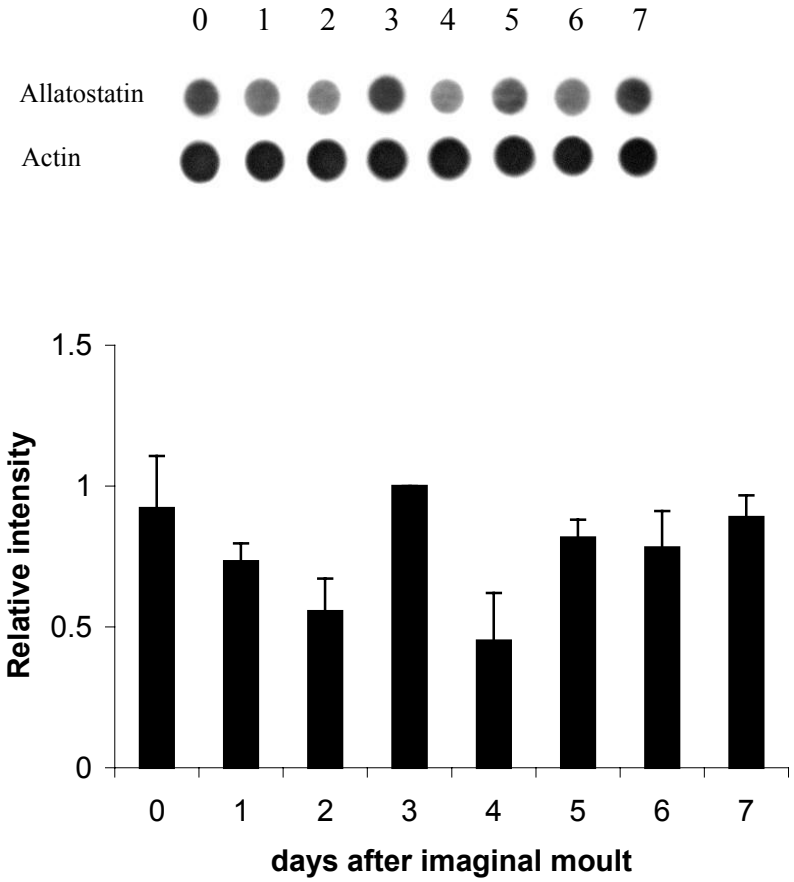

Fig. 25 RNA dot blot analysis of the allatostatin type B expression in the hindgut of adult virgin female crickets. Details are given in Fig. 20. Mean values ± SD, n = 4.

3.4.3.5. *Grybi*-AS B expression in the fat body of virgin females

Allatostatin type B expression in the fat body of virgin females was highly variable. After ecdysis, the expression increased on day 1, but decreased to day 2. After another increase on day 3 and day 4, the expression level decreased again on day 5. Another peak value appeared on days 6/7 (Fig. 26).

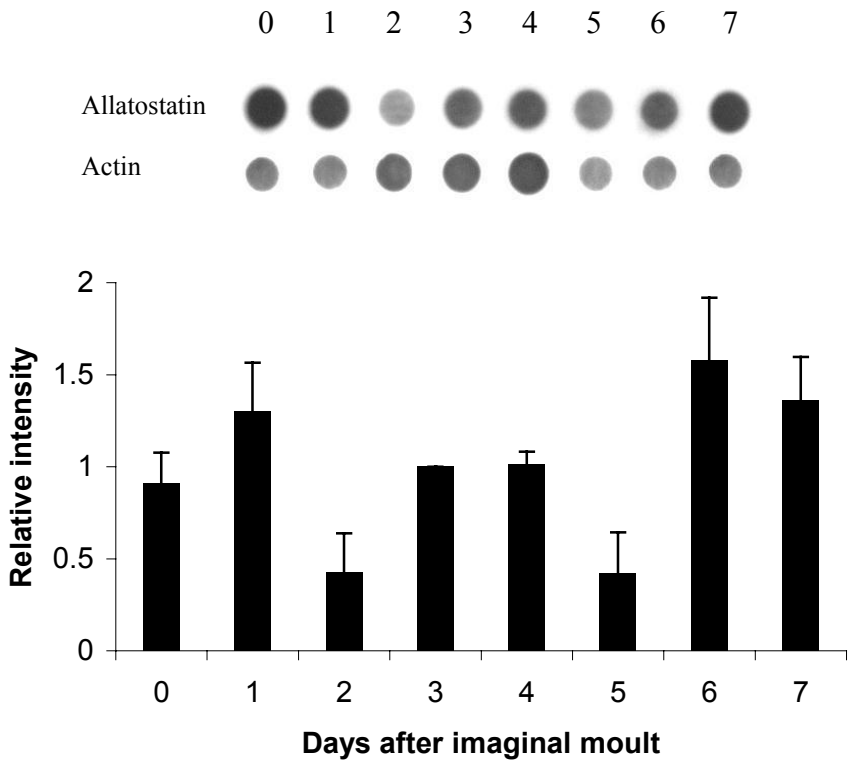

Fig. 26 RNA dot blot analysis of the allatostatin B type expression in the fat body of adult virgin female crickets. Details are given in Fig. 20. Mean values ± SD, n = 4.

3.4.3.6. *Grybi*-AS B expression in the accessory reproductive glands of males

After ecdysis, allatostatin type B expression in accessory reproductive glands of males remained fairly constant until day 2 and then decreased to day 3 (Fig. 27).

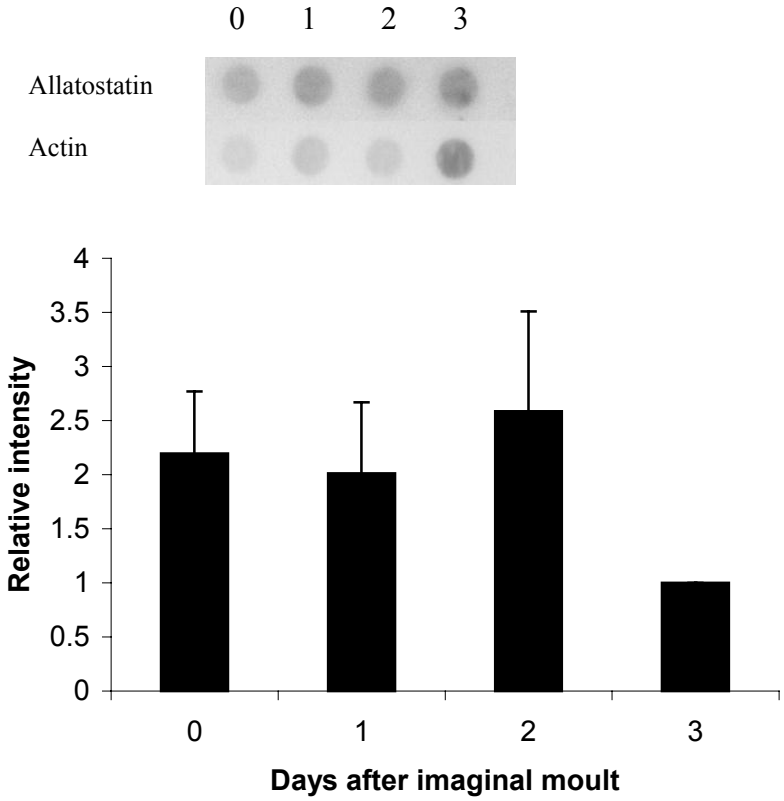

Fig. 27 RNA dot blot analysis of the allatostatin type B expression in the accessory reproductive glands of adult male crickets. Details are given in Fig. 20. Mean values ± SD, n = 4.

3.4.3.7. *Grybi*-AS B expression in the testes of crickets

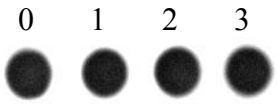

Fig. 28 RNA dot blot of the allatostatin B type expression in the testes of adult male cricket.

Allatostatin type B expression was proven in testes, but the limited amount of mRNA did not allow further analysis with replicates (Fig. 28).

4. Discussion

4.1. Structures and functions of W(X)$_6$W-allatostatins (type B allatostatins)

The partial 3´cDNA sequence of *Grybi*-AS B gene as elucidated in this work encodes four different peptides flanked by Lys-Arg endoproteolytic cleavage sites. All of the four allatostatins contain the conserved W^2W^9 structure followed by a Gly residue serving as substrate for carboxyl-amidation, which is typical for the W(X)$_6$Wamides (Williamson, 2001a; Lorenz, 2001). The amino acid sequences of Grybi-AS B1, B2, and B3 deduced from the 3´cDNA sequence are consistent with the peptides previously purified from brain extracts of cricket by conventional HPLC techniques (Lorenz et al., 1995a).

Six W(X)$_6$W-allatostatins have been identified from the cricket *G. bimaculatus* so far: Grybi-AS B1-B5 were isolated by conventional chromatographic techniques from the brains (Lorenz et al., 1995a, 1999), and a new member of the peptide family, Grybi-AS B6 was deduced from the 3´cDNA sequence. Grybi-AS B1 and B2 are highly homologous in structure, by differing in only one amino acid at position 3 (Gln/Arg). Grybi-AS B1 also has 90% amino acid sequence identity with Peram-MIP of *P. americana* (Predel et al., 2001), which differs in position 6 (Asn/Gln) from Grybi-AS B1, and 80% identity with Carmo-AS B3 from the stick insect *C. morosus* (Lorenz et al., 1998a, 2000), which differs from Grybi-AS B1 in positions 6 and 7 (Asn/Gln and Gly/Ser). Grybi-AS B4 shares the conserved C-terminal structure of Phe-His-Gly-Ser-Trp-amide with the dodecapeptides Manse-MIP 3 from *M. sexta* (Blackburn et al., 2001) and Bommo-PTSP 8 from *B. mori* (Hua et al., unpublished data; sequence submitted to Genbank accession no. AB073553). Although Grybi-AS B3 shows only low similarity in its amino acid sequence with the other cricket B type allatostins and with all other W(X)$_6$W neuropeptides, the similarity in its DNA coding sequence with *Grybi*-AS B2 is 90%, which may be caused by mutation during evolution.

So far, a total of 33 neuropeptides, all of which share the conserved W(X)$_6$W structure, were identified from *L. migratoria*, *M. sexta*, *G. bimaculatus*, *C. morosus*, *B. mori*, *D. melanogaster,* and *P. americana*. The finding of W(X)$_6$Wamides in the orders Dictyoptera, Ensifera, Caelifera, Phasmatodea, Lepidoptera, and Diptera suggests that these neuropeptides are widespread among insects, as it is the case for the FGLamide neuropeptides (A type allatostatins).

When comparing the amino acid sequences of all known W(X_6)Wamides, three identical pairs are found in *M. sexta* and *B. mori*, the nonapeptides Manse-MIP 2 and Bommo-PTSP 3 (GWQDLNSAWa), and Manse-MIP 1 and Bommo-PTSP 1 (AWQDLNSAWa), and the dodecapeptides Manse-MIP 3 and Bommo-PTSP 8 (APEKWAAFHGSWa). Manse-MIP5 (GWQDMSSAWG) and Bommo-PTSP2 (AWQDMSSAWG) differ in only one amino acid at position 1 (Gly/Ala), whereas Manse-MIP6 (AWSALHGAWG) and Bommo-PTSP5 (AWSALHGTWG) differ in one amino acid at position 8 (Ala/Thr). A difference of one amino acid also exists between Peram-MIP and Carmo-AS B3 (position 7, Gly/Ser), Carmo-AS B2 and Locmi-MIP (position 7, Thr/Ala), Locmi-MIP and Carmo-AS B5 (position 6, Asn/Gln), Bommo-PTSP 2 and Manse-MIP 5 (position 1, Ala/Gly), and Carmo-AS B4 and Carmo-AS B1 (position 8, Ala/Gly). Thirteen of the known W(X_6)Wamides share the conserved N-terminal sequence of A/GWQDL, and nine neuropeptides the conserved C-terminal sequence of SAWamide. High homology in interspecific as well as intraspecific W$(X)_6$Wamide allatostatins suggests that the genes of these neuropeptides are derived from one original gene during evolution.

In *G. bimaculatus*, the inhibiting action of the B type allatostatins on JH biosynthesis *in vitro* by the CA is rapid and reversible. However, the inhibiting potency ("potency" = effective dose for 50% inhibition = ED_{50}) of each of the peptides is different. Grybi-AS B2, B4 and B5 exhibited higher potency ED_{50} values of $1x10^{-8}$, $2x10^{-8}$ and $3x10^{-8}$ M, respectively, whereas Grybi-AS B1 and B3 showed a lower potency with an ED_{50} value of about $7x10^{-8}$ M. The locustamyoinhibiting peptide Locmi-MIP, which shares 80% amino acid sequence identity with Grybi-AS B1, showed a maximal inhibitory activity on JH biosynthesis of the CA from crickets that was nearly four orders of magnitude lower than that of Grybi-AS B1-B5 (Lorenz et al., 1995a; 1999). The B type allatostatins from *C. morosus* strongly inhibited JH biosynthesis in the CA of crickets with nearly the same potency as the Grybi-AS B peptides, but did not affect the CA of the stick insect itself (Lorenz et al., 2000).

When comparing the inhibitory action on JH biosynthesis of two types of cricket allatostatins (A type and B type), it turned out that the potency of the B type allatostatins is almost one order of magnitude lower than for the A type. It has to be considered, however, that most of the type B allatostatins occur in higher concentrations in the brain of the cricket than the A type peptides. When Grybi-AS A5 (the least active of the A type allatostatins) and Grybi-AS

B5 (a moderately active B type allatostatin) were used for a comparative study (Lorenz et al., 1995a, b; 1999), however, it turned out that the two peptides exhibited almost the same potency and efficacy in inhibiting JH release by the CA *in vitro*. No synergistic action of the two peptides could be observed.

The presence of multiple allatostatic neuropeptides in one insect species which belong to two different peptide families, the different inhibiting potency of each of the allatostatins, and the fact that no synergistic action for members of the two peptide families was observed, suggest the existence of discrete receptors at least for each type of allatostatins. The pathways of signal transduction also seem to be different. Two FGL-allatostatin (type A) receptor genes were recently cloned from *D. melanogaster* (Birgül et al., 1999; Lenz et al., 2000b, c). Receptors similar to those from *Drosophila* were also cloned from the cockroach *P. americana* (Auerswald et al., 2001) and the moth *B. mori* (Secher et al., 2001). Type A allatostatin receptor genes of *B. mori* and *D. melanogaster* have two introns which occur at exactly the same positions and have the same intron phasing, suggesting that the three receptors are not only structurally, but also evolutionarily related (Secher et al., 2001). Structurally, the transmembrane regions of the receptors are most closely related to the mammalian galanin receptors. Kreienkamp et al. (2002) identified two novel orphan G-protein coupled receptors from *D. melanogaster*, which are related to the mammalian opiod/somatostatin receptor family and were activated by a *Drosophila* C type allatostatin. However, no B type allatostatin receptors are known so far.

The $W(X)_6W$ allatostatins seem to act multifunctionally, as the other allatostatins do. In addition to their allatostatic function, B type allatostatins from crickets inhibited ovarian ecdysteroid biosynthesis, thus acting as ecdysiostatins. The inhibiting potency of each of the B type allatostatins was different, as is the case for their allatostatic function. During an *in vitro* incubation for 3 hours, ecdysteroid production was inhibited by 45% to 50% at concentrations of 10^{-7} to 10^{-9} M B1 and B2, respectively. Grybi-AS B3 and B4 were less effective in inhibiting ecdysteroid biosynthesis. A reduction in ovarian ecdysteroid biosynthesis *ex vivo* was observed in crickets after injection of Grybi-AS B. The $W(X)_6W$ neuropeptide Bommo-PTSP 1 from larval brains of *B. mori* was proved to act in a prothoracicostatic (ecdysiostatic) manner (Hua et al., 1999). Bommo-PTSP 1 inhibited ecdysteroidogenesis in the prothoracic glands (PG) of larvae in the spinning stage to a greater extent than in the PG of larvae in the feeding stage. Manse-MIP 1, which is identical to

Bommo-PTSP 1 in its amino acid sequence, is the most potent of the Manse-MIPs in inhibiting peristalsis of the ileum *in vitro*. Myoinhibiting functions were also detected in Locmi-MIP of *L. migratoria* (Schoofs et al., 1991) and in Peram-MIP of *P. americana* (Predel et al., 2001).

The high GC-content of the 3´cDNA sequence of the *Grybi*-AS B gene is surprising. The highest GC-content was found in the sequence encoding Grybi-AS B3, with 83.3%. This high GC-content probably led to the failure to isolate the 5´cDNA part of the gene sequence. When 5´RACE method was used, many clones which were truncated in this region were obtained, probably due to the poor ability of reverse transcriptase to open the secondary structures of the mRNA. Many other "hybrid" clones, which contained the known 3´sequence mismatched with upstream unrelated sequences of high GC-content, were also obtained, even under very stringent PCR conditions (data are not shown). To isolate the full length of the gene sequence, the method of construction and screening a genomic DNA library should be the first choice. Another possibility is to modify the traditional 5´RACE method by substitution of reverse transcriptase. Two polymerases can be used to substitute the traditional reverse transcriptases derived from avian myeloblastosis virus (AMV) or moloney murine leukemia virus (MMLV). One is Tth DNA polymerase, which can catalyze the polymerization of DNA using an RNA template in the presence of manganese chloride at 70°C. The ability of Tth DNA polymerase to reverse transcribe at elevated temperatures minimizes the problems encountered with strong secondary structures in RNA since they are unstable at higher reaction temperatures. Higher temperatures may also result in increased specificity of primer hybridization and extension. The limitation of Tth DNA polymerase is its processing ability of ca. 1 kb. The other one is Omniscript Reverse Transcriptase, a recombinant heterodimeric enzyme which was commercially developed by QIAGEN in July of 2003. Omniscript Reverse Transcriptase enables read-through of templates with high GC content or complex secondary structures. Limited with time and fund, these methods were not yet performed.

4.2. Spatial expression patterns of the *Grybi*-AS B gene in various tissues

4.2.1. The central nervous system

By RT *in situ* PCR, it was demonstrated that the *Grybi*-AS B gene is expressed in the median neurosecretory cells as well as in lateral neurosecretory cells and in neurons of the

protocerebrum. The strongest signals were found in the region of the median neurosecretory cells. In the deuterocerebrum and tritocerebrum, strong positive signals could be detected in various neurosecretory cells. In the suboesophageal ganglion (SOG), positive signals were found in the neurosecretory cells which surround the medulla. These results confirm the immunocytochemical distribution of Grybi-AS B type peptides. A polyclonal antiserum raised against Grybi-AS B1 was used in the peroxidase-antiperoxidase immunohistochemical technique to detect the localization of Grybi-AS B type peptides in brain, SOG, and retrocerebral complex of *G. bimaculatus* (Witek et al., 1999). In brain, immunoactivity was detected in neurosecretory cells and in PI-cells. Extensive nerve staining occurred in the whole neuropil area of the brain, in the central body, in the antennal glomeruli, in the optic nerves, in the tritocerebrum and in the calyces. In SOG, some normal and small sized cells were stained as well as the neuropil. In CC-CA complexes, immunochemical staining was observed in the CC, the nervus corpus allatum I (NCA I, connection CC and CA), and in the nervus corpus allatum II (NCA II, connecting CA and SOG) as well as in the connection between NCA I and NCA II running on the surface of the CA. Inside CC and CA, nerve branches were also stained, but no glandular cell staining could be detected.

Combining the data of RT *in situ* PCR and immunolocalization, it is suggested that the Grybi-AS B type peptides are produced in the neurosecretory cells of brain and SOG and can be delivered to the CA cells directly through nerve connections, and there act as potent inhibitors of JH biosynthesis in crickets also *in vivo*. However, they also can be released into the haemolymph through the CC as neurohaemal organ. Considering the strongest expression in the region of the median neurosecretory cells of the protocerebrum, the question rises whether the allatostains can be released into the haemolymph directly from the median neurosecretory cells? Whether allatostatins are produced in CC and CA, which are supposed as storage organs for allatostatins, is still unknown. Hopefully, this question will be disclosed by the ongoing experiments of RT *in situ* PCR in the retrocerebral complexes. *Grybi*-AS B gene expression in neurons of the brain suggests a neuromodulatory function of B type allatostatins, as it is the case for the FGL-allatostatins (Stay et al., 1992). Interestingly, different intensities of *Grybi*-AS B gene expression were observed in the median neurosecretory cells and in neurons of the protocerebrum. In the region of the median neurosecretory cells, more than one hundred cells were intensively stained, whereas inside the neurons only small granules could be detected. These results suggest that the expression can be regulated precisely on the transcription (mRNA) level. Immunocytochemical distribution of Grybi-AS A type peptides

in brain, SOG, and retrocerebral complex of *G. bimaculatus* was demonstrated to be almost identical with the expression pattern for the Grybi-AS B type peptides, suggesting that both families of allatostatins may have the same or at least similar sites and mechanisms of production and delivery.

In the cortex of thoracic ganglia, *Grybi*-AS B gene expression was localized not only in the cytoplasm of neurosecretory cells, but also in some nuclei of neurosecretory cells. In addition, in frontal and terminal abdominal ganglia, *Grybi*-AS B gene expression was detected in the neurosecretory cells of the cortex. These results suggest that Grybi-AS B type peptides may act allatostatic or in a neuromodulatory way on the associated organs or tissues which are innervated by neurons originating from thoracic ganglia or from the abdominal ganglia.

Many tissues and organs which are innervated by FGL-allatostatin-immunoreactive neurons have been found in cockroaches, mainly in the midgut, hindgut, midgut neurosecretory cells, the antennal heart, the prothoracic, salivary and male accessory glands, the abdominal perisympathetic organs (see Weaver et al., 1998), and in oviduct muscles (Woodhead et al., 2003). However, not so much immunohistochemical data are available for the $W(X)_6$Wamide neuropeptides.

4.2.2. The reproductive system and the fat body

In addition to the brain, the ovary seems to be an important organ which regulates CA activity in both a stimulatory and an inhibitory manner (Lanzrein et al., 1981; Stay et al., 1994b, 1996; Bylemans et al., 1998; Unnithan et al., 1998). The direct effect of an allatostatic ovary-derived factor on CA activity was first reported in *L. migratoria* (Gadot et al., 1987) and led to the elucidation of the peptide nature of an ovarian allatostatic factor in (Ferenz and Aden, 1993). Mature ovaries of *G. bimaculatus* contain various humoral factors which either stimulate or inhibit the biosynthesis of JH in the CA (Hoffmann et al., 1996).

In the cricket ovary, each panoistic ovariole consists of a distal germarium and a more proximal vitellarium. These two regions reflect two phases of oocyte growth and development. The germarium contains the stem line oogonia and prefollicular tissue and their derivatives. The stem line oogonia are derived from the original germ cells. When they divide, one of the daughter cells remains a functional stem line cell, while the other becomes a definitive

oogonium and develops into an oocyte. During this developmental stage, oocyte is regulated directly by the its genome and contains species-specific information. Oocytes pass down the ovariole and enlarge in size. Their nucleus also increases in size (now known as the germinal vesicle). Transcription of the nuclear DNA appears to be suppressed soon after the beginning of yolk uptake. During yolk deposition, as the oocyte grows much more rapidly, the germinal vesicle is relatively smaller and, finally, the nuclear membrane breaks down. As each oocyte leaves the germarium, it is clothed by the prefollicular tissue which form the follicular epithelium. By cell division, the follicular epithelium keeps pace with oocyte growth. During yolk accumulation, the follicular cells, which surround the rapidly growing oocyte, no longer divide and become stretched over the oocyte as a flattened, squamous epithelium. The follicular cells may be binucleate or endopolyploid caused by continuous nuclear division even without cell division, permitting the high levels of synthetic activity in which the cells are involved. In the vitellarium, the oocyte growth is mainly regulated by genes outside the oocyte, producing pools of molecules that will subsequently be involved in embryonic growth (Chapman, 1998).

In the germarium, *Grybi*-AS B gene expression was localized in the nuclei of primary oocytes. Condensed signals were detected in the nucleus of the young primary oocytes near the terminal filament, but no signals were detected in the prefollicular cells. These results suggest that B type allatostatins are intensely involved in the early-stage development of oocytes in crickets. With the ongoing development of oocytes, the signals in the nucleus became weaker. Eventually, no signals could be detected in the nucleus (germinal vesicles) of vitellogenic oocytes, whereas the signals were now detected in the follicular cells. The changes of temporal and spatial gene expression are accordant with mechanisms regulating the entire development of the oocytes.

In the vitellarium, the RT *in situ* PCR positive signals were detected in the follicular cells surrounding each oocyte. As expected, no signals were detected in the germinal vesicles nor in the cytoplasm of the oocytes considering the collapse of the nuclear membrane. Existence of B type allatostatins in the cytoplasm of oocytes from *G. bimaculatus* was demonstrated by immunohistochemical methods, as it was demonstrated for FGL-allatostatins (type A) in *G. bimaculatus* (Witek et al., 2001) and *D. punctata* (Woodhead et al., 2003). However, for any allatostatins, presence of peptides or their prohormones in the follicular cells was not detected. Combining these results, it was supposed that the synthesis of Grybi-AS type B peptides in

the follicular cells may be regulated precisely on the translation (protein) level. After transcription the switch-on of translation is induced by specific signal(s). Thereafter, peptides may be released into the cytoplasm of the oocyte. Another possibility is, that the allatostatins synthesized in the follicular cells are immediately released into the haemolymph.

The follicular cells are very important for the growth and development of oocytes. The functions of the follicular cells change during oocyte development. In the early stage, the follicular cells produce some minor yolk protein, ecdysone or a precursor of ecdysone, and perhaps some enzymes that may be involved in processing the yolk at a later time. The follicular cells are also involved in the uptake of vitellogenins from the haemolymph into the oocytes, which is facilitated by calmodulin. The intercellular spaces of the follicular epithelium permit direct access of the haemolymph to the surface of the oocyte. The uptake of proteins from the hemolymph is regulated by JH. In the later stages of oogenesis, the follicular cells produce the vitelline envelope and the ligands responsible for the determination of the terminals of the embryo and its dorso-ventral axis. In the last stage, they produce the egg shell or chorion for ovulation. Expression of the *Grybi*-AS B gene localized in follicular cells suggests that a possible ecdysiostatic function of the peptides may exist also *in vivo*, and as it has been demonstrated *in vitro*. Grybi-AS type B peptides may also affect the development of oocytes by their allatostatic effect on JH biosynthesis.

Some other results of allatostatins present in the ovary were reported. By RT-PCR, it was demonstrated that the FGL-allatostatin gene was expressed not only in ovaries but also in oviducts of *D. punctata*. However, immunoactivity was only detected in nerves branching on the surface of the oviduct (Garside et al., 2002). Very recently, in *D. punctata*, it was reported that FGL-allatostatin immunoactivity is evident in the oocyte cytoplasm, at the micropyle and on the surface of the chorion, in the lumen of the follicle cell sheath, in the lumen of the lateral oviduct below the ovary, in epithelial cells of the oviduct, in nerves leading to the muscles of the oviduct, and in yolk cells. The authors also suggested that FGL-allatostatins in the oocyte/oviduct, may function in the process of ovulation, and after ovulation, allatostatins in the fertilized egg may function in yolk utilization (Woodhead et al., 2003).

In fat body, most yolk protein is synthesized independently of the ovaries. In addition to vitellogenins and lipophorin, some insects are known to produce smaller amounts of other (yolk) proteins in the fat body. In most insects, vitellogenin synthesis is initiated by JH acting

directly on the fat body. RT *in situ* PCR positive signals were detected in the fat body, suggesting that Grybi-AS type B peptides may be involved in vitellogenin synthesis and release of the proteins by a feed-back regulation of JH synthesis. In *G. bimaculatus*, vitellogenin titers in the haemolymph of allatostatin-injected animals were almost twice as high as in controls, but no significant effect on the haemolymph JH titers could be detected, suggesting allatostatins might inhibit the vitellogenin uptake by the follicular cells (Lorenz et al., 1998b). In cockroaches A type allatostatins inhibit vitellogenin production (Martin et al., 1996) and the release from the periovarian fat body tissue (Martin et al., 1998).

Positive RT *in situ* PCR signals were also detected in the epithelium cells of accessory reproductive glands (ARG) of female crickest, suggesting Grybi-AS B type peptides may be involved in regulating some enzymes associated with ARG secretions. In cockroaches, FGL-allatostatin immunoactivity was detected in the neurons innervating the male accessory glands.

4.2.3. The digestive and absorption system

Cells of the caecum and midgut epithelium are actively involved in enzyme production and secretion, as well as in absorption of nutrients. In caecum and midgut, *Grybi*-AS B gene expression was localized in endocrine secretory cells, suggesting that Grybi-AS type B peptides may influence the activity of some enzymes involved in digestion of carbohydrates or proteins. In *D. punctata*, the FGL-allatostatin Dippu-AS 7 was found to stimulate activity of both invertase and α-amylase in a dose-dependent manner in the lumen of ligatured midguts *in vitro* (Fusé et al., 1999).

In the hindgut, positive RT *in situ* PCR signals were detected in the gut epithelium cells and in both longitudinal and circular muscles, suggesting possible myoinhibiting functions of Grybi-AS B petides on visceral muscles, as it was shown for other $W(X)_6W$ peptides from *L. migratoria*, *L. maderae*, *P. americana* and *M. sexta*.

4.2.4. The thoracic muscles

Grybi-AS B gene expression was detected in thoracic muscles of the cricket. This result suggests that Grybi-AS type B peptides may act in a myoinhibiting way on flight muscles.

4.3. Comparison of RT *in situ* PCR results with the dot blot analysis

The results of RT *in situ* PCR in different tissues were confirmed by RT-PCR and RNA dot blot. For each tissue investigated by RT *in situ* PCR, positive signals were also obtained by RT-PCR and RNA dot blot, indicating the results of RT *in situ* PCR are reliable.

In this work, the RT *in situ* PCR was proved to be an effective method for localization of expression of insect neuropeptide genes in various tissues. The method of *in situ* hybridization was also used, and similar results were obtained, with tissues such as of ovary, caecum and midgut (data are not shown). Compared to the RT *in situ* PCR, the sensitivity of *in situ* hybridization was lower (at least in this study). In some tissues such as muscles and hindgut where cuticle are abundantly present, the method of *in situ* hybridization is hardly applicable due to a robust unspecific absorption of colour-substrate by cuticle. For a successful use of RT *in situ* PCR, the step of protease digestion is critical. Less protease digestion will lead to insufficient degradation of genomic DNA, which subsequently will cause false positive signals. Over-digestion will destroy the tissues and cell structures. The protease digestion times have to be different for different tissues. For each tissue sample, a series of protease digestion over different time periods had be done to determine the optimal protease digestion time.

The aim of RNA dot blot analysis was to reveal a putative age-dependent relationship between *Grybi*-AS B gene expression and JH biosynthesis. However, results of RT *in situ* PCR in different tissues suggested that Grybi-AS type B peptides act multifunctionally during the development of the crickets through rather complex pathways. Limited by the understanding of the complex regulation mechanisms, and also limited by the methodological sensitivity, no clear relationships between *Grybi*-AS B gene expression and JH biosynthesis could be elucidated from the RNA dot blot analyses.

4.4. Future aspects

To elucidate the regulation mechanisms of $W(X)_6W$ allatostatins in crickets, the identification of putative receptor(s) is necessary. Some FGL-allatostatin receptors have been identified from *D. punctata*, *D. melanogaster, B. mori,* and *P. americana*. With the help of established

genome datebases of the fruit fly, silk worm and mammals, the isolation of putative receptor(s) for Grybi-AS type B peptides seems promising.

Functional genomic analysis may contribute to a comprehensive understanding of the regulatory mechanisms of $W(X)_6W$ allatostatin action in crickets. However, functional analysis of genes in *Gryllus* has been hindered by the absence of genetic transformation techniques. An attempt to establish a transgenic system in *G. bimaculatus* was recently reported (Zhang et al., 2002). Using class II transposable elements which are functional in a wide range of insects as tools for germline transformation, the promoter sequence of *Gryllus* cytoplasmic actin was isolated and subcloned upstreamly to the reporter gene of GFP (green fluorescent protein), and then transformed to *Gryllus* embryos (eggs) by injection. Examination of transformants indicated that gene expression of GFP driven by *Gryllus* cytoplasmic actin promoter could be detected in extraembryonic cells of vitellophages, but not in the germ-band. However, this transgenic system seems to be limited to this developmental stage.

Double-stranded RNA-mediated interference (RNAi) has recently emerged as a powerful reverse genetic tool to silence gene expression. RNAi is an evolutionarily conserved phenomenon and a multistep process that involves generation of active small interfering RNA (siRNA) *in vivo* through the action of an RNase III endonuclease, Dicer. The resulting 21- to 23-nt siRNA mediates degradation of the complementary homologous RNA. By this mechanism, double-stranded RNA (dsRNA) can trigger silencing of homologous gene expression (reviewed in Bernstein et al., 2001; Sharp, 2001; Zamore, 2001). RNAi can be applied in organisms without a transgenic system using synthesized double-stranded RNA. Using RNAi to functionally knockout the *Grybi*-AS B genes, associated with accurate physiological and biochemical examinations, will promisingly contribute to elucidate the regulatory mechanisms of Grybi-AS type B peptides. This attempt is ongoing in our laboratory.

5. Summary

1. Cricket B type allatostatins, which belong to a neuropeptide family sharing the conserved $W(X)_6$Wamide structure, exhibited inhibitory functions on the biosynthesis of juvenile hormones (JH) *in vitro* in the corpora allata (CA) as well as on ecdysteroid biosynthesis in the ovary of adult crickets (*Gryllus bimaculatus*). To understand the mechanisms of function of the pleiotropic cricket B type allatostatins, it is necessary to characterize their gene (preprohormone) and study the spatial and temporal expression patterns of the gene.

2. By PCR screening of a random primer cDNA library and by RACE (Rapid Amplification of cDNA Ends), a 535 bp 3´cDNA sequence of the cricket B type allatostatin gene was yielded. This 3´cDNA fragment encodes a putative translation product of 85 amino acids with potential dibasic endoproteolytic cleavage sites, which may allow processing into six peptides including three copies of Grybi-AS B1 (GWQDLNGGWGa) and single copies of Grybi-AS B2 (GWRDLNGGWGa), Grybi-AS B3 (AWRDLSGGWGa), and Grybi-AS B6 (AWNNLGSAWGa), respectively. Three of these deduced peptides were previously isolated from cricket brain extracts by conventional chromatographic techniques and were designated as Grybi-AS B1, Grybi-AS B2, and Grybi-AS B3. The Grybi-AS B6 neuropeptide represents a novel member of the B type allatostatins.

3. The nucleotide sequences encoding the type B allatostatins are high in GC-content and show strong homology. The highest GC-content was found for *Grybi*-AS B3 with 83.3%. The similarity of the nucleotide sequences encoding *Grybi*-AS B2 and *Grybi*-AS B1 is 93.3%, whereas *Grybi*-AS B2 and B3 share 90% nucleotide identity.

4. By Southern blot analyses, it was proven that the *Grybi*-AS type B gene is present as a single copy per haploid genome of *G. bimaculatus*.

5. By RT *in situ* PCR technique, it could be demonstrated that the *Grybi*-AS B gene is expressed in various tissues of 1 day old female adult crickets:

   - In the central nervous system

     Grybi-AS B gene expression was detected in the brain. In the protocerebrum, strong positive signals were found in the median neurosecretory cells, and to a lesser extent

in lateral neurosecretory cells and in neurons. Gene expression was also found in the neurosecretory cells of the deuterocerebrum and the tritocerebrum. Furthermore, *Grybi* - AS B gene expression was localized in neurosecretory cells of the suboesophageal ganglion (SOG), the thoracic ganglia, and the abdominal ganglia.

- In the reproductive system

  In the germarium and in primary oocytes of the ovary, *Grybi*-AS B gene expression was detected as condensed signals in the nuclei, but not in the prefollicular cells or the cytoplasm. With ongoing development of the oocytes, the signals in the nuclei (germinal vesicles) appeared as separated granules with weaker intensity, which finally disappeared, whereas in the follicular cells strong signals became apparent.

  Grybi-AS B gene expression was also detected in the epithelial cells of the accessory reproductive glands of female crickets.

- In the digestive and absorption system

  In caecum and midgut, *Grybi*-AS B gene expression was found in endocrine secretory and epithelial cells, whereas in the hindgut, positive RT *in situ* PCR signals were detected in both longitudinal and circular muscles and in the gut epithelial cells.

- In other tissues

  Grybi-AS B gene expression was also found in cells of the fat body and in thoracic (flight) muscles.

6. The results on *Grybi*-AS B gene expression as obtained by RT *in situ* PCR were confirmed by RT-PCR and RNA dot blot analyses. The expression of the *Grybi*-AS B gene in various tissues of adult females varied in an age-dependent manner. In brains of virgin females gene expression increased from the day of emergence until day 8 of adult life. In the ovary of virgin females gene expression showed a maximum at day 4 after ecdysis, whereas in mated females gene expression was high during the first two days and at days 6 to 7, but low inbetween. In caecum and midgut of virgin females gene expression was low during the first 5 days after ecdysis, but peaked at days 6 and 7, whereas in the hindgut gene expression was highest at day 3 of adult life. In the fat body, gene expression showed highest values on day 1 and days 6 to 7 after ecdysis.

7. Gene expression in brain, testes, and accessory reproductive glands of 0 to 3 days old male crickets was also demonstrated by RT-PCR and RNA dot blot analyses.

6. Zusammenfassung

1. Die Allatostatine vom Typ B bilden eine Familie von Neuropeptiden [W(X_6)Wamide], die bei Grillen (*Gryllus bimaculatus*) die Biosynthese von Juvenilhormonen (JH) *in vitro* in den Corpora allata (CA) hemmen, aber auch die Synthese von Ecdysteroiden (Häutungshormonen) in den Ovarien. Um die Funktionen dieser pleiotropen Neuropeptide besser zu verstehen, ist es notwendig, ihr Gen bzw. Präprohormon zu charakterisieren und die räumlichen und zeitlichen Muster der Expression zu studieren.

2. Mittels PCR Screening einer cDNA Bibliothek und RACE (Rapid Amplification of cDNA Ends) ist es gelungen, eine 535 Basenpaare lange 3´cDNA-Sequenz des Grillen Typ B Allatostatin-Gens zu erhalten. Dieses 3´cDNA Fragment kodiert für ein 85 Aminosäuren langes Translationsprodukt, das zahlreiche dibasische, endoproteolytische Schnittstellen enthält, welche die Prozessierung von sechs Peptiden ermöglichen: drei Kopien des Neuropeptids Grybi-AS B1 (GWQDLNGGWGa) und je eine Kopie der drei Neuropeptide Grybi-AS B2 (GWRDLNGGWGa), Grybi-AS B3 (AWRDLSGGWGa) und Grybi-AS B6 (AWNNLGSAWGa). Drei dieser Peptide waren in einer früheren Untersuchung unserer Arbeitsgruppe aus methanolischen Gehirnextrakten von Grillen mittels konventioneller chromatographischer Techniken isoliert worden und erhielten die Bezeichnungen Grybi-AS B1, Grybi-AS B2 und Grybi-AS B3. Das Neuropeptid Grybi-AS B6 stellt ein neues Mitglied der Typ B Allatostatin-Familie dar.

3. Die die Typ B Allatostatine kodierenden Nukleotidsequenzen sind ausgesprochen GC-reich und weisen eine hohe Homologie auf. Der höchste GC-Gehalt wurde bei *Grybi*-AS B3 mit 83,3% gefunden. *Grybi*-AS B2 und *Grybi*-AS B1 weisen in ihrer Nukleotidsequenz 93,3% Homologie auf, *Grybi*-AS B2 und *Grybi*-AS B3 sind zu 90% identisch.

4. Mittels Southern Blot konnte gezeigt werden, dass das Allatostatin Typ B Gen bei *Gryllus bimaculatus* in nur einer Kopie pro haploidem Genom vorliegt.

5. Mittels der RT *in situ* PCR Technik wurde gezeigt, dass das *Grybi*-AS B Gen in verschiedenen Geweben von 1-Tag alten adulten Grillenweibchen exprimiert wird:

- Im Zentralnervensystem

  Im Protocerebrum des Gehirns wurde ein besonders starkes positives Signal in den medianen neurosekretorischenn Zellen gefunden und etwas geringere Reaktion in den lateralen neurosekretorischen Zellen und in Neuronen. Genexpression fand auch in den neurosekretorischen Zellen des Deuterocerebrums und des Tritocerebrums statt. Weiterhin wurde Genexpression in den neurosekretorischen Zellen des Suboesophagealganglions (SOG), der Thorakalganglien und der Abdominalganglien lokalisiert.

- In den Fortpflanzungsorganen

  Im Germarium und in den primären Oozyten des Ovars wurde Genexpression in Form eines kondensierten Signals in den Zellkernen gefunden, aber nicht im Zytoplasma der Oozyten und in den Präfollikelzellen. Während der Oogenese ändert das Signal im Kern seine Form (getrennte Granulae) und wird schwächer, um schließlich ganz zu verschwinden. Zu diesem Zeitpunkt findet man aber ein starkes Expressionssignal in den reifen Follikelzellen. *Grybi*-AS B Genexpression wurde auch in den Epithelzellen der akzessorischen Geschlechtsdrüsen nachgewiesen.

- Im Verdauungstrakt

  In den Darmblindsäcken und im Mitteldarm wurde eine starke Genexpression in endokrinen sekretorischen Zellen und Epithelzellen gefunden, wohingegen im Enddarm das RT *in situ* PCR Signal in Längs- und Ringmuskulatur sowie in den Epithelzellen lokalisiert war.

- In anderen Geweben

  Grybi-AS B Genexpression konnte darüber hinaus in Zellen des Fettkörpers und in der Thorax(Flug-)muskulatur nachgewiesen werden.

6. Die Ergebnisse aus den Genexpressionsstudien mittels RT *in situ* PCR wurden durch RT-PCR und RNA Dot-blot Analysen bestätigt. Die Expression des *Grybi*-AS B Gens zeigte in allen untersuchten Geweben von adulten Grillenweibchen einen altersabhängigen Verlauf. Im Gehirn von unbegatteten Weibchen stieg die Genexpression vom Tag der Imaginalhäutung bis zum 8. Tag des Adultlebens kontinuierlich an. In den Ovarien

unbegatteter Weibchen zeigte die Genexpression am 4. Tag nach der Adulthäutung ein Maximum, während die Expression im Ovar begatteter Weibchen während der ersten beiden Tage nach der Häutung hoch war, dann abfiel und erst zu den Tagen 6/7 hin wieder anstieg. In den Darmblindsäcken und im Mitteldarm konnte in den ersten fünf Tagen nach der Häutung nur eine geringe Expression festgestellt werden, aber ein Anstieg bei den älteren Tieren (Tage 6/7); im Enddarm war die Expression am 3. Tag nach Ecdysis am höchsten. Im Fettkörper erreichte die Expression am ersten Tag des Adultlebens sowie an den Tagen 6 und 7 Maximalwerte.

7. Auch in verschiedenen Geweben (Gehirn, Hoden, Akzessorische Geschlechtsdrüsen) von 1- bis 3-Tage alten adulten Männchen konnte mittels RT-PCR und RNA Dot-blot Analyse *Grybi*-AS Typ B Genexpression festgestellt werden.

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Erklärung

Hiermit erkläre ich, daß ich die Arbeit selbständig verfaßt und keine anderen als die von mir angegebenen Quellen und Hilftsmittel benutzt habe.

Ferner erkläre ich, daß ich nicht anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

Junling Wang

Bayreuth, den 21.01.2004