Determination of allatostatin levels in relation to the gonadotropic cycle in the female of *Blattella germanica* (L.) (Dictyoptera, Blattellidae)

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Abstract. A polyclonal antibody against the allatostatin BLAST-3 (AGSDGRLYSFGL-NH$_2$) of the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae) has been raised and characterized, and an ELISA (enzyme-linked immunosorbent assay) for allatostatin quantification has been developed. Allatostatin contents in brain, midgut and haemolymph have been measured in females of *B. germanica* during the first gonadotropic cycle. Brain allatostatin content increases steadily from adult emergence to the formation of the first ootheca. The values range from 2 ng/brain on the day of adult emergence to 25 ng/brain when the insect forms the ootheca 8 days later. In the midgut, the pattern is similar but the values are about half those of the brain. Allatostatin concentrations in the haemolymph after HPLC separation are in the nanomolar range. The occurrence of allatostatins in the haemolymph suggests that these peptides can act through a humoral pathway, as well as via nerves. The allatostatin content of both brain and midgut are high while the female is transporting the ootheca, which suggests that these peptides could be related to the low metabolic status characterising the period of oothecal transport.

Key words. Allatostatin, *Blattella germanica*, brain, ELISA, haemolymph, midgut.

Introduction

Pleiotropy is a common feature in complex endocrine systems. Allatostatins, for example, are insect peptides showing a typical YXFGL-NH$_2$ C-terminal sequence, that were identified initially by their inhibitory activity on Juvenile Hormone (JH) production in cockroaches (Pratt *et al.*, 1989; Woodhead *et al.*, 1989), although they also elicit antimyotropic activity in the gut (see for example Duve *et al.*, 1995a; Lange *et al.*, 1995) and inhibitory properties over vitellogenin release by the fat body (Martín *et al.*, 1996). Peptides belonging to the allatostatin family have been identified in cockroaches (Pratt *et al.*, 1989, 1991; Woodhead *et al.*, 1989, 1994; Bellés *et al.*, 1994; Weaver *et al.*, 1994), flies (Duve *et al.*, 1993, 1994,

1995b, 1996), mosquitoes (Veenstra *et al.*, 1997), crickets (Lorenz *et al.*, 1995), locusts (Veelaert *et al.*, 1996a,b), moths (Davis *et al.*, 1997; Duve *et al.*, 1997a,b) and in a crab (Duve *et al.*, 1997c). In addition to peptide identification, cDNAs (Donly *et al.*, 1993; Ding *et al.*, 1995; Vanden Broeck *et al.*, 1996; Bendena *et al.*, 1997; Veenstra *et al.*, 1997; Bellés *et al.*, 1999) or genes (East *et al.*, 1996) encoding for allatostatins have been cloned and sequenced.

In the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae), four allatostatins have been identified up to now (Bellés *et al.*, 1994), and up to thirteen have been deduced from the cDNA codifying for the corresponding prohormone (Bellés *et al.*, 1999). The occurrence of allatostatin immunoreactive material detected by immunocytochemical techniques in different areas of the central nervous system, stomatogastric nervous system and gut of *B. germanica* (Maestro *et al.*, 1998), suggests that there must be undescribed functions for allatostatins in this cockroach. An approach that may provide clues to unveil these possible new functions would be to

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quantify allatostatin levels in different tissues and in key physiological situations. With this aim, we have raised an antibody against one of the *B. germanica* allatostatins and, on the basis of this antibody, we have developed an enzyme-linked immunosorbent assay (ELISA) to measure allatostatin levels in brain, midgut and haemolymph of *B. germanica* females on selected days within the first gonadotropic cycle and the period of oothecal transport.

Materials and Methods

Insects

Adult females of *B. germanica* were obtained from a colony fed on dog chow and water, and reared in the dark at $30 \pm 1°C$ and 60–70% relative humidity. Freshly moulted virgin females were isolated and used at the appropriate physiological ages, which were assessed by measuring the basal oocyte length (Bellés *et al.*, 1987). In general, virgin specimens were used, but the experiments with females in the period of oothecal transport were carried out with mated specimens, because they retain the ootheca during the whole embryogenesis. In these females, the presence of spermatozoa in the spermathecae was assessed, thus indicating that mating had occurred.

Bioactive compounds and preparation of conjugates

Allatostatins used in the present study were those identified up to now in *B. germanica*, BLAST-1: LYDFGL-NH$_2$, BLAST-2: DRLYSFGL-NH$_2$, BLAST-3: AGSDGRLYSFGL-NH$_2$, and BLAST-4: APSSAQRLYGFGL-NH$_2$ (Bellés *et al.*, 1994). The peptides were synthesized in an Abimed AMS 422 multiple synthesiser (Abimed, Analysentechnik, Langenfeld, Germany) using Fmoc chemistry. To raise an antibody against BLAST-3, the peptide was conjugated to keyhole-limpet haemocyanin (KLH) (Sigma, Madrid, Spain) according to Sambrook *et al.* (1989). BLAST-3 was also conjugated to chicken ovalbumin (Sigma) for coating the ELISA plates for the first step in ELISA development. Optimal concentration of BLAST-3-ovalbumin, determined according to Muñoz *et al.* (1986), was found to be 0.45 μg/ml.

Antibody production and titre test

The antibody against BLAST-3 was raised in three male New Zealand white rabbits. The rabbits were injected subcutaneously on days 0, 7 and 14 with 75 μg of peptide (in the conjugated form) diluted in 500 μl of phosphate-buffered saline (PBS) (0.2 M, pH 7.2) and emulsified with an equal volume of Freund's complete adjuvant (Sigma) on day 0, or incomplete adjuvant on days 7 and 14. Blood samples were obtained on day 21. Then, rabbits were boosted again once a month for 7 months, using the same dose and incomplete adjuvant, and serum was obtained one week after each booster injection. A titre curve using dilutions of anti-BLAST-3 serum from 1:2000 to 1:64,000, was tested against a constant concentration of BLAST-3-ovalbumin conjugate, determined as described above. Half-maximum antiallatostatin binding was obtained at a dilution of 1:9000, this value being considered the antibody titre.

ELISA development

BLAST-3 conjugated to chicken ovalbumin (Sigma) was coated on wells of 96-well ELISA plates (NUNC-Immuno Plate Maxisorp 96F, Roskilde, Denmark) in carbonate buffer (0.05 M, pH 9.6, 100 μl/well) and incubated for 2 h at room temperature. BLAST-3-ovalbumin conjugate was not added to blank control wells. Then the solution was discarded and the wells were washed in PBS-0.05% Tween three times, and in PBS twice more. The plate was then blocked with 3% bovine serum albumin (Sigma), in carbonate buffer (100 μl/well) and incubated overnight at 4°C. After washing, 50 μl of the sample were added to wells and 11 dilutions of synthetic BLAST-3 (from 9.77 pg to 10 ng) were used for a standard curve. Immediately afterwards, 50 μl of the anti-BLAST-3 serum (1:4500 diluted in PBS), was added to each well. Plates were incubated for 2 h at room temperature, with gentle shaking. After washing, 100 μl of the secondary antiserum goat anti-rabbit IgG conjugated to peroxidase (Sigma, diluted in PBS at 1:10 000) was added to the wells, which were then incubated for 2 h at room temperature. Finally, wells were washed again and substrate solution was added (100 μl/well) and incubated for 8 min in the dark with gentle shaking. Substrate solution was prepared with 12.5 ml of citrate buffer (pH 5), 200 μl of 0.6% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide and 50 μl of 1% H$_2$O$_2$. The colour reaction was stopped by adding 75 μl of 2 N H$_2$SO$_4$ to each well. Absorbance was read to 450 nm with a Titertek Multiscan Plus MKII spectrophotometer (Labsystems, Helsinki, Finland).

Sample preparation

Brains and midguts were dissected under Ringer solution and homogenized in PBS. Haemolymph samples were obtained by cutting off one leg of the animal and applying gentle pressure to the abdomen. The haemolymph was collected using a micropipette and diluted in PBS. Then all three tissues were processed in the same way. Samples were boiled for 5 min and centrifuged at 16 000 *g* for 10 min. Supernatants were collected and the pellets were re-extracted, centrifuged again and the supernatants were pooled with the previous ones and stored at −20°C.

In a first series of measurements, samples were subjected to solid phase extraction. They were diluted in water with 0.1% trifluoroacetic acid (TFA), loaded onto C$_{18}$ Isolute (IST, U.K.) cartridges and eluted with 17%, 40% and 100% CH$_3$CN with 0.1% TFA. Brain, midgut and haemolymph extracts of females at different ages, and different concentrations of synthetic BLAST-3 were fractionated and assayed by ELISA. Results corresponding to the extract of one brain equivalent from 4-

day-old females were 0.68 ± 0.06 ng, 4.81 ± 0.65 ng and 0.93 ± 0.06 ng (mean $\pm$ SEM, $n = 4$) for the fractions corresponding to 0–17%, 17–40% and 40–10% CH₃CN/TFA, respectively. Those corresponding to midgut and haemolymph extracts gave similar results, that is, the bulk (70–80%) of BLAST-3 immunoreactivity appeared in the 17–40% fraction, as expected according to our previous analytical data (Bellés *et al.*, 1994). This, and the fact that recovery of synthetic peptide after solid phase extraction was lower than 50% (the processing of 1.25 ng BLAST-3 gave 0.58 ± 0.09 ng, mean $\pm$ SEM, $n = 3$, in the 17–40% fraction), led us to use crude extracts for current determination of BLAST-3 immunoreactivity in tissues.

HPLC studies

For HPLC studies, haemolymph samples were extracted, pooled together, diluted in water with 0.1% TFA and processed in a Merck–Hitachi low-pressure system with automatic gradient controller (L-6200A) and UV-VIS detector (L-4200). The column was a C_{18} LiChrospher (Merck, Darmstadt, Germany) (4×125 mm, 5 μm particle size), supplied with a guard column using the same material. The separations were performed as described previously (Bellés *et al.*, 1994), using a gradient of H_2O/CH_3CN, both solvents having 0.1% TFA, and at a flow rate of 1 ml/min. The gradient used was 0–8% of CH_3CN in 6 min and then 40% in 64 min. Using synthetic BLAST-3 as standard, the recovery was higher than 95%. Two-minute fractions were collected and assayed in the ELISA. In the above conditions, synthetic peptides BLAST-1, -2, -3 and -4, eluted between 42 and 46 min.

Results

ELISA characterization

A typical sensitivity curve (Fig. 1) was obtained with the immunoreactivities elicited by different concentrations of synthetic BLAST-3 (9.77 pg–10 ng) using the anti-BLAST-3 serum at a dilution of 1 : 9000. The sigmoidal curve was subjected to a logit transformation (Fig. 1, inset) using the ratio B-T/B_0-T, where B_0 is the maximal optical density corresponding to the absence of competition, B is the optical density corresponding to the samples, and T is the optical density of nonspecific binding. This transformation showed that values from 150 pg to 5 ng of peptide fitted well into a linear regression ($r = -0.98$). The point $y = 0$ of this curve corresponds to a value of $x = 850$ pg of BLAST-3, which is the amount of antigen at the half-maximal binding (B-T/B_0-T = 0.5), that is the optimal sensitivity of the method.

In order to study the selectivity of the antibody, cross-reactivity assays of anti-BLAST-3 serum with synthetic BLAST-1, -2 and -4 were carried out. Series of different concentrations of BLAST-1, -2 and -4 were compared with the results obtained when BLAST-3 was used, and an ED_{50} (ng of peptide required for 50% displacement of binding to conjugate) was calculated. The reactivity, corresponding to the $ED_{50} \times 100$ for each allatostatin relative to BLAST-3, was 8.17% for BLAST-1, 6.26% for BLAST-2 and 1.18% for BLAST-4.

Levels of allatostatin estimated from crude extracts of brain, midgut and haemolymph

BLAST-3 levels were quantified by ELISA in extracts of individual brains and midguts and in haemolymph of virgin females during the first gonadotropic cycle. The profile of BLAST-3 immunoreactivity levels in brain (Fig. 2) shows that they are low until day 2, then increase from day 2–5, and remain stabilized from day 5–7, giving values between 20 and 25 ng/brain. The formation of the ootheca occurs on day 8, and 24 h after the ootheca formation, BLAST-3 immunoreactivity still increases. However, if the ootheca is artificially removed 24 h after its formation, and allatostatin levels are determined 4 days later, when the female starts vitellogenesis and the length of their basal oocytes is 0.90 ± 0.07 mm (mean $\pm$ SEM, $n = 11$), then a significant (*t*-test, $P = 0.01$) decrease in brain BLAST-3 levels is observed.

Levels of BLAST-3 in the midgut (Fig. 2) start to rise from day 1 and maintain a steady increase throughout the gonadotropic cycle until day 7, and later during the first and second day of the oothecal transport period. The profile obtained almost parallels that of the levels in the brain, although values for the midgut are about a half of those of the brain. Again, if allatostatin levels in the midgut are measured 4 days after removal of the ootheca, they show a significant (*t*-test, $P = 0.04$) decrease.

BLAST-3 immunoreactivity in crude haemolymph was also estimated using the same extraction procedure and assay. The profile obtained during the gonadotropic cycle was also approximately parallel to that corresponding to brain and midgut. However, the values measured in the haemolymph were within the micromolar range, concentrations that were considered too high for what might be expected for a circulating hormone, thus prompting the studies described under the next heading.

Allatostatin levels estimated from extracts purified by HPLC

To clarify whether the high levels of allatostatins measured in the haemolymph were due to a certain proportion of non-specific immunoreactivity, we decided to perform an HPLC separation of haemolymph pooled from 3- and 6-day-old females. Using the system described in the Material and Methods, the synthetic *Blattella* allatostatins eluted between 42 and 46 min. For each run, a volume between 500 and 940 μl of pooled haemolymph was used, and immunoreactivity measured in fractions between 42–46 min was 4.62 ± 1.09 pg/μl (mean $\pm$ SEM, $n = 2$) in samples from 3-day-old females, and 7.98 ± 2.85 pg/μl (mean $\pm$ SEM, $n = 3$) in 6-day-old females. We also detected significant levels of immunoreactivity in
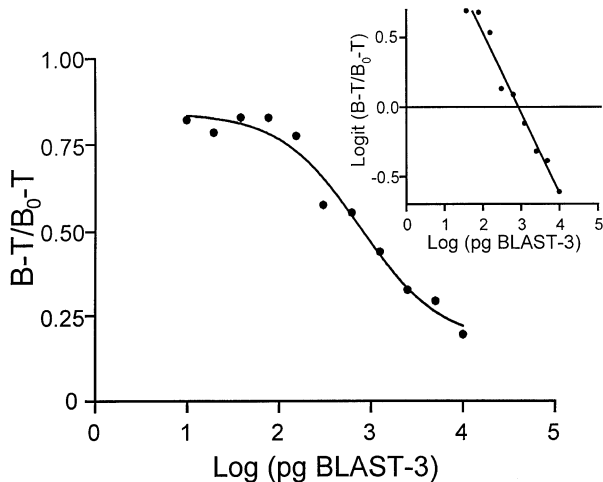
Fig. 1. Sensitivity curve of *Blattella germanica* anti-BLAST-3 serum (diluted 1: 9000) obtained with increasing amounts of synthetic BLAST-3 from 9.77 pg to 10 ng. The inset shows the logit transformation of this curve. The point $y = 0$ represents the optimal sensitivity value that corresponds to a $x = 850$ pg of BLAST-3, which is the amount of antigen at the half-maximal binding (B-T/B_0-T = 0.5).
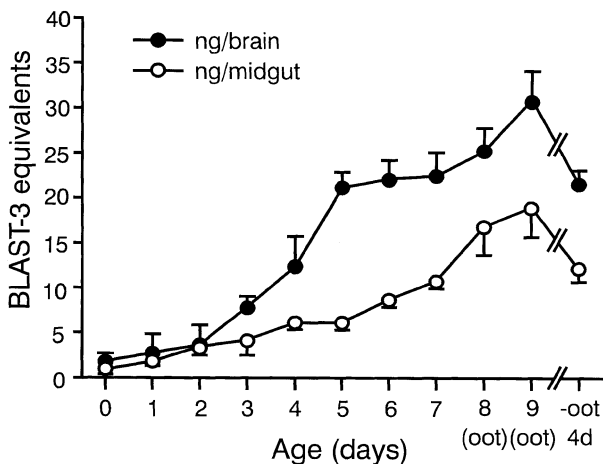
Fig. 2. Content of BLAST-3 equivalents in brain and midgut from *Blattella germanica* females throughout the first gonadotropic cycle. (oot): females transporting the ootheca. –oot 4d: females 4 days after removal of the ootheca. Results expressed as the mean ± SEM ($n = 9–11$).

fractions between 48–50 min (1.93 ± 0.42 pg/µl, mean ± SEM, $n = 2$, for 3-day-old females; 5.19 ± 1.52 pg/µl, mean ± SEM, $n = 3$, for 6-day-old females) and 52–56 (2.61 ± 0.68 pg/µl, mean ± SEM, $n = 2$, for 3-day-old females; 6.78 ± 2.62 pg/µl, mean ± SEM, $n = 3$, for 6-day-old females), which are clearly out of the retention time range of these peptides in our HPLC conditions. This apparently non-specific immunoreactivity
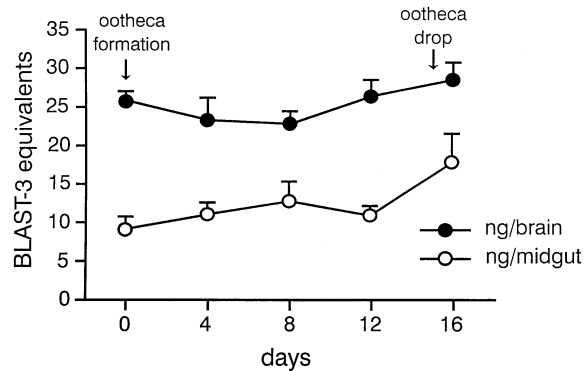
Fig. 3. Content of BLAST-3 equivalents in brain and midgut from *Blattella germanica* females in selected days of the oothecal transport period. The ootheca was dropped between days 14 and 16, when (or just before) the young emerged. Results expressed as the mean ± SEM ($n = 7–10$).

accounts partly for the high values obtained from crude or solid phase extracted haemolymph samples.

In the light of these results, we also carried out an HPLC separation of a pool of fifty brains from 5-day-old females. In this case, immunoreactivity appeared in fractions from 42–46 min, as expected, whereas no immunoreactivity was observed between 48–56 min. The estimated amount of BLAST-3 equivalents was 6.86 ng/brain, a value which is within the same order of magnitude of those measured in crude extracts of brain (Fig. 2).

Brain and midgut allatostatin levels during oothecal transport

To study whether the high levels of BLAST-3 immunoreactivity observed after oviposition are maintained while the female is transporting the ootheca, brain and midgut extracts were tested from mated females on different days within the period of oothecal transport. ELISA measurements were carried out 0, 4, 8 and 12 days after the ootheca formation. Allatostatin levels were also tested in females between 10 and 48 h after the ootheca was dropped, that is, 16 days after the ootheca formation. These females had started a second gonadotropic cycle, having a basal oocyte measuring 1.13 ± 0.06 mm (mean ± SEM, $n = 7$). Allatostatin levels in the brain (Fig. 3) on the day of ootheca formation were high, around 25 ng/brain, and remained at this level on days 4, 8 and 12 within the period of oothecal transport, and also on day 16, when the ootheca had been just dropped. The comparison of levels between different days (0, 4, 8, 12 and 16) did not show statistically significant differences.

Levels of BLAST-3 in midgut (Fig. 3) followed a similar pattern, the results showing sustained high values (around 12 ng/midgut) during the period of oothecal transport. Also in this case the levels found on different days were not statistically different from each other.

Discussion

An ELISA using an antibody raised against the allatostatin BLAST-3 of *B. germanica* has been developed and characterized. The range of detection has allowed the detection of BLAST-3 immunoreactivity levels in crude extracts of individual brains or midguts. Previous reports (Yu *et al*., 1993; Reichwald *et al*., 1994) had described ELISA methods to quantify allatostatins in the cockroach *Diploptera punctata*. In comparison with ours, the ELISAs described by these two groups show better sensitivity levels, which could be explained by the use of different detection systems: the biotin/streptavidin reaction by Yu *et al*. (1993), and the enzyme alkaline phosphatase by Reichwald *et al*. (1994). However, the tetramethylbenzydine–peroxidase method used by us is simpler and cheaper than that of biotin/streptavidin, and faster than that of alkaline phosphatase, and the sensitivity is sufficient to measure levels in individual brains or midguts of our model species. The low cross-reactivity of the BLAST-3 antibody when tested against the other three known allatostatins of *B. germanica*, BLAST-1, -2 and -4, suggests that the immunoreactivity detected in a given tissue is mainly due to the peptide BLAST-3.

BLAST-3 immunoreactivity in brain and midgut show similar qualitative patterns during the first gonadotropic cycle. In both cases, allatostatin levels increase steadily from days 0 to 7 (Fig. 2). In the cockroach *D. punctata*, Yu *et al*. (1993) reported that the minimum content of allatostatin in the female brain coincides with the highest level of JH production, while a subsequent decrease in JH production corresponds with an increase in brain allatostatins. In *B. germanica* the profile of allatostatin in the female brain does not show this inverse correlation with JH production. Indeed, the moment of maximal activity of the corpora allata (CA) is on day 6 (Maestro *et al*., 1994), when brain allatostatin content is high (Fig. 2). Nevertheless, in both cockroaches, *B. germanica* and *D. punctata*, allatostatin contents in brain are high around the time of oviposition, when JH levels are remarkably low (Fig. 2, and Yu *et al*., 1993).

The allatostatin content of the midgut throughout the first gonadotropic cycle follows a profile similar to that found in the brain, although the amounts measured in the midgut are about a half those of the brain. This is also the case in *D. punctata*, as estimated by Reichwald *et al*. (1994) with biological assays. In *B. germanica*, immunocytochemical techniques revealed the occurrence of allatostatin-like material in axons innervating the midgut coming from perikarya located in the brain and in the last abdominal ganglion, as well as in endocrine cells of the midgut epithelium (Maestro *et al*., 1998). The function of these cells may be related to the detection of the nutrient content in the gut for modulating enzyme secretion or gut motility (Duve & Thorpe, 1994; Duve *et al*., 1995a; Maestro *et al*., 1998). In insects belonging to different orders, it has been reported that allatostatins inhibit the contraction of different areas of the gut (Duve & Thorpe, 1994; Duve *et al*., 1995a, 1996; Lange *et al*., 1995, 1997a).

BLAST-3 immunoreactivity in the haemolymph was also tested during the first gonadotropic cycle. Both crude and solid phase extracts of haemolymph showed very high values, much higher than could be expected for a peptide hormone. For example, in *D. punctata*, haemolymph concentration of *Diploptera* allatostatin 7 assayed by ELISA after solid phase extraction was never higher than 2.5 nM (Yu *et al*., 1993). The high values measured in haemolymph extracts, either crude or subjected to solid phase extraction, led us to further separate the samples by HPLC. Fractions from HPLC were assayed with the ELISA, but immunoreactivity was found not only at the retention times corresponding to BLAST-1, -2, -3 and -4, but also in fractions eluting later, out of the typical retention time corresponding to allatostatins. Conversely, when the same HPLC–ELISA procedure was applied to brain extracts, immunoreactivity was detected only in the fractions corresponding to allatostatins. The results suggest that ELISA applied to crude haemolymph reveals significant levels of apparently non-specific immunoreactivity. In any case, BLAST-3 immunoreactivity values obtained after HPLC separation of haemolymph extracts from 3- and 6-day-old females suggest that the allatostatin concentration is in the nanomolar range, and increases during the vitellogenic period also in that tissue, which is in agreement with the patterns found in brain and midgut (Fig. 2).

Allatostatin levels were also tested in relation to the formation of the ootheca. They were high, in both brain and midgut, at the time of oviposition and even increased one day later (Fig. 2). When the ootheca was removed, thus prompting a new gonadotropic cycle (Osorio *et al*., 1998), allatostatin levels had significantly decreased 4 days later, although they were still higher than those observed in females in the first gonadotropic cycle having the same length of basal oocyte (3–4-day-old females) (Fig. 2). Measurements carried out every 4 days during the period of oothecal transport suggested that high concentrations of allatostatin are maintained until the end of this period (Fig. 3). In the viviparous species *D. punctata*, Yu *et al*. (1993) reported that concentration of brain allatostatins is high at the beginning of the period of embryo transport, but decreases gradually towards the end of that period. This difference can be due to the more modified reproductive strategy of *D. punctata*.

In *B. germanica* Osorio *et al*. (1998) have reported that the presence of the ootheca in the genital atrium inhibits feeding directly, and CA development and JH production indirectly. They also proposed the hypothesis that allatostatins could be the factor that inhibits CA development under conditions of low food consumption (Osorio *et al*., 1998). The high levels of allatostatins measured during the period of oothecal transport, which is also characterized by a low food consumption (see below), seems to reinforce the above hypothesis. Indeed, during the period of oothecal transport, *B. germanica* shows a peculiar physiological status, characterized by low rates of JH production (Gadot *et al*., 1989; Maestro *et al*., 1994), low locomotory activities (Lee & Wu, 1994) or low food consumption (Lee & Wu, 1994; Osorio *et al*., 1998). The activity of allatostatins inhibiting JH production by the CA (Bellés *et al*., 1994), or vitellogenin release by the fat body (Martín *et al*., 1996), or, in other cockroaches, as antimyotropic agents acting in different areas of the digestive system

(*D. punctata*: Lange *et al.*, 1995; *Leucophaea maderae*: Duve *et al.*, 1995a), seems related to this status of low metabolism and low general activity characterising the period of oothecal transport.

Our results suggest that *B. germanica* must produce high levels of allatostatin during the period of oothecal transport to maintain this status quo that arrests gonadotropic activities and allows the retention of the ootheca in the genital atrium. It seems that the development of a sustained 'depressing' action of allatostatins during a period functionally equivalent to pregnancy may have been a key step in the evolution towards viviparity in cockroaches.

Acknowledgements

References

Bellés, X., Casas, J., Messeguer, A. & Piulachs, M.D. (1987) *In vitro* biosynthesis of JH III by the corpora allata of adult females of *Blattella germanica* (L.). *Insect Biochemistry*, **17**, 1007–1010.

Bellés, X., Graham, L.A., Bendena, W.G., Ding, Q., Edwards, J.P., Weaver, R.J. & Tobe, S.S. (1999) The molecular evolution of the allatostatin precursor in cockroaches. *Peptides*, **20**, 11–22.

Bellés, X., Maestro, J.L., Piulachs, M.D., Johnsen, A.H., Duve, H. & Thorpe, A. (1994) Allatostatic neuropeptides from the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). Identification, immunolocalization and activity. *Regulatory Peptides*, **53**, 237–247.

Bendena, W.G., Garside, C.S., Yu, C.G. & Tobe, S.S. (1997) Allatostatins: diversity in structure and function of an insect neuropeptide family. *Neuropeptides in Development and Aging. Annals of the New York Academy of Sciences*, **814**, 53–66.

Davis, N.T., Veenstra, J.A., Feyereisen, R. & Hildebrand, J.G. (1997) Allatostatin-like-immunoreactive neurons of the tobacco hornworm, *Manduca sexta*, and isolation and identification of a new neuropeptide related to cockroach allatostatins. *Journal of Comparative Neurology*, **385**, 265–284.

Ding, Q., Donly, B.C., Tobe, S.S. & Bendena, W.G. (1995) Comparison of the allatostatin neuropeptide precursors in the distantly related cockroaches *Periplaneta americana* and *Diploptera punctata*. *European Journal of Biochemistry*, **234**, 737–746.

Donly, B.C., Ding, Q., Tobe, S.S. & Bendena, W.G. (1993) Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach *Diploptera punctata*. *Proceedings of the National Academy of Sciences of the U.S.A.*, **90**, 8807–8811.

Duve, H., Johnsen, A.H., Maestro, J.L., Scott, A.J., Crook, N., Winstanley, D. & Thorpe, A. (1997a) Identification, tissue localisation and physiological effect *in vitro* of a neuroendocrine peptide identical to a dipteran Leu-callatostatin in the codling moth *Cydia pomonella* (Tortricidae: Lepidoptera). *Cell and Tissue Research*, **289**, 73–83.

Duve, H., Johnsen, A.H., Maestro, J.L., Scott, A.G., East, P.D. & Thorpe, A. (1996) Identification of the dipteran Leu-callatostatin peptide family: the pattern of precursor processing revealed by

isolation studies in *Calliphora vomitoria*. *Regulatory Peptides*, **67**, 11–19.

Duve, H., Johnsen, A.H., Maestro, J.L., Scott, A.G., Jaros, P.P. & Thorpe, A. (1997c) Isolation and identification of multiple neuropeptides of the allatostatin superfamily in the shore crab *Carcinus maenas*. *European Journal of Biochemistry*, **250**, 727–734.

Duve, H., Johnsen, A.H., Maestro, J.L., Scott, A.G., Winstanley, D., Davey, M., East, P.D. & Thorpe, A. (1997b) Lepidopteran peptides of the allatostatin superfamily. *Peptides*, **18**, 1301–1309.

Duve, H., Johnsen, A.H., Scott, A.G., East, P. & Thorpe, A. (1994) [Hyp3]Met-callatostatin. Identification and biological properties of a novel neuropeptide from the blowfly *Calliphora vomitoria*. *Journal of Biological Chemistry*, **269**, 21059–21066.

Duve, H., Johnsen, A.H., Scott, A.G., East, P. & Thorpe, A. (1995b) Isolation, identification and functional significance of [Hyp^2]Met-callatostatin and des Gly-Pro Met-callatostatin, two further post-translational modifications of the blowfly neuropeptide Met-callatostatin. *Regulatory Peptides*, **57**, 237–245.

Duve, H., Johnsen, A.H., Scott, A.G., Yu, C.G., Yagi, K.J., Tobe, S.S. & Thorpe, A. (1993) Callatostatins: neuropeptides from the blowfly *Calliphora vomitoria* with sequence homology to cockroach allatostatins. *Proceedings of the National Academy of Sciences of the USA*, **90**, 2456–2460.

Duve, H. & Thorpe, A. (1994) Distribution and functional significance of Leu-callatostatins in the blowfly *Calliphora vomitoria*. *Cell and Tissue Research*, **276**, 367–379.

Duve, H., Wren, P. & Thorpe, A. (1995a) Innervation of the foregut of the cockroach *Leucophaea maderae* and inhibition of spontaneous contractile activity by callatostatin neuropeptides. *Physiological Entomology*, **20**, 33–44.

East, P., Tregenza, K., Duve, H. & Thorpe, A. (1996). Identification of the dipteran Leu-callatostatin peptide family: characterisation of the prohormone gene from *Calliphora vomitoria* and *Lucilia cuprina*. *Regulatory Peptides*, **67**, 1–9.

Gadot, M., Burns, E. & Schal, C. (1989) Juvenile hormone biosynthesis and oocyte development in adult female *Blattella germanica*: effects of grouping and mating. *Archives of Insect Biochemistry and Physiology*, **11**, 189–200.

Lange, A.B., Bendena, W.G. & Tobe, S.S. (1995) The effect of the thirteen Dip-Allatostatins on myogenic and induced contractions of the cockroach (*Diploptera punctata*) hindgut. *Journal of Insect Physiology*, **41**, 581–588.

Lee, H.-J. & Wu, Y.-L. (1994) Mating effects on the feeding and locomotion of the German cockroach, *Blattella germanica*. *Physiological Entomology*, **19**, 39–45.

Lorenz, M.W., Kellner, R. & Hoffmann, K.H. (1995) Identification of two allatostatins from the cricket, *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae): additional members of a family of neuropeptides inhibiting juvenile hormone biosynthesis. *Regulatory Peptides*, **57**, 227–236.

Maestro, J.L., Bellés, X., Piulachs, M.D., Thorpe, A. & Duve, H. (1998) Localization of allatostatin-immunoreactive material in the central nervous system, stomatogastric nervous system, and gut of the cockroach *Blattella germanica*. *Archives of Insect Biochemistry and Physiology*, **37**, 269–282.

Maestro, J.L., Danés, M.D., Piulachs, M.D., Cassier, P. & Bellés, X. (1994) Juvenile hormone inhibition in corpora allata from ovariectomized *Blattella germanica*. *Physiological Entomology*, **19**, 342–348.

Martín, D., Piulachs, M.D. & Bellés, X. (1996) Inhibition of vitellogenin production by allatostatin in the German cockroach. *Molecular and Cellular Endocrinology*, **121**, 191–196.

Muñoz, C., Nieto, A., Gayá, A., Martínez. J. & Vives, J. (1986) New experimental criteria for optimization of solid-phase antigen concentration and stability in ELISA. *Journal of Immunology Methods*, **94**, 137–144.

Osorio, S., Piulachs, M.D. & Bellés, X. (1998) Feeding and activation of corpora allata in the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *Journal of Insect Physiology*, **44**, 31–38.

Pratt, G.E., Farnsworth, D.E., Fok, K.F., Siegel, N.R., McCormack, A.L., Shabanowitz, J., Hunt, D.F. & Feyereisen, R. (1991) Identity of a second type of allatostatin from cockroach brains: an octadecapeptide amide with a tyrosine-rich address sequence. *Proceedings of the National Academy of Sciences of the USA*, **88**, 2412–2416.

Pratt, G.E., Farnsworth, D.E., Siegel, N.R., Fok, K.F. & Feyereisen, R. (1989) Identification of an allatostatin from adult *Diploptera punctata. Biochemical and Biophysical Research Communications*, **163**, 1243–1247.

Reichwald, K., Unnithan, G.C., Davis, N.T., Agricola, H. & Feyereisen, R. (1994) Expression of the allatostatin gene in endocrine cells of the cockroach midgut. *Proceedings of the National Academy of Sciences of the USA*, **91**, 11894–11898.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Vanden Broeck, J., Veelaert, D., Bendena, W.G., De Tobe, S.S. & Loof, A. (1996) Molecular cloning of the precursor cDNA for schistostatins, locust allatostatin-like peptides with myoinhibiting properties. *Molecular and Cellular Endocrinology*, **122**, 191–198.

Veelaert, D., Devreese, B., Schoofs, L., Van Beeumen, J., Vanden Broeck, J., De Tobe, S.S. & Loof, A. (1996a) Isolation and characterization of eight myoinhibiting peptides from the desert locust, *Schistocerca gregaria*: new members of the cockroach allatostatin family. *Molecular and Cellular Endocrinology*, **122**, 183–190.

Veelaert, D., Devreese, B., Van den Broeck, J., Yu, C.G., Schoofs, L., Van Beeumen, J., De Tobe, S.S. & Loof, A. (1996b) Isolation and characterization of schistostatin-2 (11–18) from the desert locust, *Schistocerca gregaria*: a truncated analog of schistostatin-2. *Regulatory Peptides*, **67**, 195–199.

Veenstra, J.A., Noriega, F.G., Graf, R. & Feyereisen, R. (1997) Identification of three allatostatins and their cDNA from the mosquito *Aedes aegypti. Peptides*, **18**, 937–942.

Weaver, R.J., Freeman, Z.A., Pickering, M.G. & Edwards, J.P. (1994) Identification of two allatostatins from the CNS of the cockroach *Periplaneta americana*: novel members of a family of neuropeptide inhibitors of insect juvenile hormone biosynthesis. *Comparative Biochemistry and Physiology*, **107C**, 119–127.

Woodhead, A.P., Khan, M.A., Stay, B. & Tobe, S.S. (1994) Two new allatostatins from the brains of *Diploptera punctata. Insect Biochemistry and Molecular Biology*, **24**, 257–263.

Woodhead, A.P., Stay, B., Seidel, S.L., Khan, M.A. & Tobe, S.S. (1989) Primary structure of four allatostatins: neuropeptide inhibitors of juvenile hormone synthesis. *Proceedings of the National Academy of Sciences of the USA*, **86**, 5977–6001.

Yu, C.G., Stay, B., Joshi, S. & Tobe, S.S. (1993) Allatostatin content of brain, corpora allata and haemolymph at different developmental stages of the cockroach, *Diploptera punctata*: quantitation by ELISA and bioassay. *Journal of Insect Physiology*, **39**, 111–122.