Elsevier Editorial System(tm) for Insect Biochemistry and Molecular Biology
Manuscript Draft

Manuscript Number: IB-D-09-00209R2

Title: Juvenile hormone and allatostatins in the German cockroach embryo

Article Type: Full Length Article

Keywords: Juvenile hormone, Corpora allata, Allatostatin, Insect embryogenesis, Blattella germanica, methyl farnesoate epoxidase

Corresponding Author: Dr. Jose L. Maestro, Ph. D.

Corresponding Author's Institution: Institut de Biologia Evolutiva (CSIC-UPF)

First Author: Jose L. Maestro, Ph. D.

Order of Authors: Jose L. Maestro, Ph. D.; Nuria Pascual, Dr.; Karl Treiblmayr, Dr.; Jesús Lozano; Xavier Bellés, Dr

Abstract: Levels of juvenile hormone III (JH), FGLamide allatostatin peptides (ASTs), ASTs precursor (preproAST) mRNA and methyl farnesoate epoxidase (CYP15A1) mRNA were measured in embryos of the cockroach Blattella germanica. JH starts to rise just after dorsal closure, reaches maximal levels between 60% and 80% of embryogenesis, and decrease subsequently to undetectable levels. ASTs show low levels during the first two thirds of embryogenesis, increase thereafter and maintain high levels until hatching. PreproAST mRNA shows quite high levels during the two days following oviposition, thus behaving as a maternal transcript, the levels then become very low until mid embryogenesis, and increase afterwards, peaking towards the end of embryo development. CYP15A1 transcripts were detected around 25% embryogenesis and the levels tended to increase through embryogenesis, although differences amongst the days studied were not statistically significant. The opposite patterns of JH and AST towards the end of embryo development, along with the detection of AST immunoreactivity in corpora allata from late embryos, suggest that JH decline is caused by the increase of AST. Moreover, the uncorrelated patterns of JH concentration and CYP15A1 mRNA levels suggest that CYP15A1 expression does not modulate JH production.

Juvenile hormone and allatostatins in the German cockroach embryo

José L. Maestro[a*], Núria Pascual[a,b], Karl Treiblmayr[c], Jesús Lozano[a], Xavier Bellés[a*]

[a] Institut de Biologia Evolutiva (CSIC-UPF), Passeig Marítim de la Barceloneta 37-49. 08003 Barcelona, Spain.

[b] Present address: Applied Molecular Receptors Group (IQAC, CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain.

[c] University of Salzburg, Department of Organismic Biology, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria.

[*] Corresponding authors. J.L. Maestro: Tel. +34 932309634; fax +34 932309555; e-mail address joseluis.maestro@ibe.upf-csic.es. X. Bellés: Tel. +34 932309636; fax. +34 932309555; e-mail address xavier.belles@ibe.upf-csic.es.

Abstract

Levels of juvenile hormone III (JH), FGLamide allatostatin peptides (ASTs), ASTs precursor (preproAST) mRNA and methyl farnesoate epoxidase (CYP15A1) mRNA were measured in embryos of the cockroach *Blattella germanica*. JH starts to rise just after dorsal closure, reaches maximal levels between 60% and 80% of embryogenesis, and decrease subsequently to undetectable levels. ASTs show low levels during the first two thirds of embryogenesis, increase thereafter and maintain high levels until hatching. PreproAST mRNA shows quite high levels during the two days following oviposition, thus behaving as a maternal transcript, the levels then become very low until mid embryogenesis, and increase afterwards, peaking towards the end of embryo development. CYP15A1 transcripts were detected around 25% embryogenesis and the levels tended to increase through embryogenesis, although differences amongst the days studied were not statistically significant. The opposite patterns of JH and AST towards the end of embryo development, along with the detection of AST immunoreactivity in corpora allata from late embryos, suggest that JH decline is caused by the increase of AST. Moreover, the uncorrelated patterns of JH concentration and CYP15A1 mRNA levels suggest that CYP15A1 expression does not modulate JH production.

Keywords: Juvenile hormone, Corpora allata, Allatostatin, Insect embryogenesis, Blattella germanica, methyl farnesoate epoxidase

1. Introduction

Juvenile hormones (JHs) are sesquiterpenoids produced by the retrocerebral glands called corpora allata (CA), and play a pivotal role in the regulation of insect development, metamorphosis and reproduction. Patterns of JH production have been established in a number of insects and the regulation of JH synthesis has been extensively studied. In this context, neuropeptides eliciting inhibitory (allatostatins) or stimulatory (allatotropins) effects on CA activity have been reported in a number of insect species (Stay, 2000; Stay & Tobe, 2007). Three types of allatostatins have been isolated in insects: FGLamide-type, $W(X)_6$Wamide-type and PISCF-type, which show different group specificities (Stay & Tobe, 2007). The FGLamide-type (herein called ASTs for brevity) has been reported in a number of insect and crustacean species, but they inhibit JH synthesis only in cockroaches, crickets and termites (Stay & Tobe, 2007).

In the cockroach *Blattella germanica*, haemolymph JH concentration has been reported for penultimate and last nymphal instars, as well as for adult females (Treiblmayr et al., 2006). The patterns suggest the function of JH as determinant of nymphal molts in juvenile stages and as gonadotrophic hormone in the adult female. Four ASTs (BLAST-1 to -4) have been identified from *B. germanica* brain extracts (Bellés et al., 1994), and the cDNA sequence of the AST precursor (preproAST) has revealed the occurrence of 9 additional putative AST peptides (Bellés et al., 1999). AST levels, in terms of both peptide and mRNA, have been determined in brain and midgut tissues of *B. germanica* adult females (Vilaplana et al., 1999; Aguilar et al., 2003), and their activity as inhibitors of JH production by adult female CA has been established

(Bellés et al., 1994). All these goals were achieved using nymphs or adults, but no information about JH and ASTs has been reported in *B. germanica* embryos.

JH titer has been measured in embryos of the cockroaches *Nauphoeta cinerea* (Lanzrein et al., 1985) and *Blatta orientalis* (Short & Edwards, 1992). In addition, JH production has been reported for CA from embryos of the cockroach *Diploptera punctata* (Holbrook et al., 1996; 1998; Stay et al., 2002). In all cases, JH levels were very low or undetectable until embryo dorsal closure, and then increased quite suddenly and decreased thereafter until hatching. This decrease may have an important physiological role and might be tightly regulated, at least in hemimetabolan insects, given that treatment of late embryos of the cricket *Acheta domesticus* with JH or the JH mimic pyriproxifen, disrupts normal development (Erezyilmaz et al., 2004). The present work aims at studying whether ASTs might contribute to regulate the decrease of JH occurring in late embryogenesis in the hemimetabolan model *B. germanica*.

2. Materials and Methods

2.1. Insects

Adult specimens of *B. germanica* were obtained from a colony fed on dog chow and water, and reared in the dark at 30 ± 1ºC and 60-70% relative humidity. Adult males and females were placed together to promote mating. In this cockroach, eggs (between 35 and 40 per gonadotrophic cycle) are packaged into a rigid egg-case or ootheca, which is transported by the female attached to the genital atrium until egg hatching. For embryo studies the ootheca was removed from the female at chosen

stages, and then the presence of spermatozoa was checked in the spermathecae in order to assess that the female was mated.

2.2. Identification and quantification of juvenile hormone in embryos

Methodology for the identification and quantification of JH has been previously described (Rembold & Lackner, 1985; Treiblmayr et al., 2006). Staged embryos were dissected and pooled in groups of 4 to 8, and then extracted in 2 ml methanol containing 0.5 pmol ethyl ester homologues of JH I and JH III as internal standards. The method is based on extraction in iso-octane, adsorption-desorption sequences involving derivatization of JHs and measurement by combined gas chromatography-selected ion monitoring mass spectrometry (GC-MS-SIM). Measurements were carried out with an Agilent GC 6890 N gas chromatograph coupled with an Agilent MSD 5973 mass detector. An Agilent 7683 autosampler was used for injection into the gas chromatograph. The temperature of the injection port was 300ºC, the initial temperature of the oven was 100ºC, and oven program increased the temperature from 100ºC to final 300ºC (30ºC/min). A fused silica capillary column (J&W DB 1, film thickness 0.25 µm, length 30 m, 0.25 µm ID) was used to separate the JH derivatives, and the limit of detection was 0.02 pmol of JH. It is worth noting that the method does not measure MF, since it is based on methoxyhydrin derivatives, which implies presence of an epoxide group (Rembold & Lackner, 1985; Treiblmayr et al., 2006).

2.3. Sample preparation and allatostatin quantification

Oothecae of known ages were removed and boiled for 10 min. Then, 10 embryos from each ootheca were homogenized in PBS, centrifuged, and the supernatant collected and lyophilized. AST measurements using competitive ELISA based on BLAST-3 peptide of *B. germanica* (AGSDGRLYSFGL-NH_2) were performed as previously described (Vilaplana et al., 1999). Briefly, BLAST-3-ovoalbumin was used as conjugated, and the same peptide in its unconjugated form was used as standard. The secondary antibody was anti-rabbit conjugated to peroxidase (Sigma), while the substrate solution was prepared in citrate buffer (pH 5), with 3,3',5,5'-tetramethylbenzidine and H_2O_2. Results are expressed as BLAST-3 equivalents (Vilaplana et al., 1999; Maestro & Bellés, 2006).

2.4. Cloning of CYP15A1 cDNA

Degenerate primers based on mRNA conserved regions of the cytochrome P450 CYP15A1 (methyl farnesoate epoxidase) sequences from different hemimetabolan species (*Diploptera punctata*, GenBank accession number: AY509244; *Reticulotermes flavipes*: FJ792774; *Acyrthosiphon pisum*: XM_001945899 and XM_001952585; *Pediculus humanus*: XM_002423030), were used to obtain a fragment of *B. germanica* CYP15A1 homologue, using a cDNA from 4- to 6-day-old adult female CA as template. Using the primer sequences 5'-TTTGATGGMMGACCHGATGG-3' and 5'-AANACYTCRGGRTCWCCCCART-3', a 952-bp fragment of *B. germanica* CYP15A1 was obtained, which was subcloned into pSTBlue-1 vector (Novagen) and sequenced.

2.5. RNA isolation, cDNA preparation, polymerase chain reaction (PCR) and Southern blot analysis

RT-PCR followed by Southern blotting was used to determine mRNA expression pattern of preproAST, as previously described (Maestro & Bellés, 2006; Maestro et al., 2009), although in the present case RNA extraction from whole oothecae was performed using RNeasy Plant kit from Qiagen. Equal amounts (300 ng) of total RNA from staged embryos were used for synthesizing cDNA. To estimate mRNA levels, a non-saturating number of cycles in the PCR system was followed. PreproAST transcript was amplified with a primer pair described previously (Maestro & Bellés, 2006). As a reference, the same cDNAs were amplified with a primer pair specific for *B. germanica* actin 5C, as described (Maestro et al., 2005). Southern blot analysis was carried out using cDNA probes generated by PCR with the same primer pairs, and labelled with fluorescein using Gene Images random prime-labelling module (Amersham Biosciences). RT-PCR followed by Southern blotting of total RNA without reverse transcription did not result in amplification, thus indicating that there was no genomic contamination.

Tissue specificity of CYP15A1 expression was tested on CA, muscle, fat body, ovary, colleterial gland and midgut tissues of 3- to 4-day-old adult females. cDNA was made using total RNA from 3 CA pairs, whereas, in the case of the other tissues, 500 ng of total RNA were used. CYP15A1 transcript was amplified using primers available upon request. The absence of genomic contamination was checked as above.

2.6. Real-Time PCR analyses

The mRNA expression levels of *B. germanica* preproAST, CYP15A1 and actin-5C were analysed using real-time PCR. The corresponding cDNA was obtained as described above, and cDNA levels in the different samples were quantified by using iQ SYBR Green supermix (Bio-Rad) in an iQ cycler and iQ single colour detection system (Bio-Rad). Primer sequences are available upon request. Total reaction volume was 20 µl. All reactions were run in duplicate or triplicate. The program used to amplify the reaction was as follows: (i) 95˚C for 3 min; (ii) 95˚C for 10 sec; (iii) 60˚C for 1 min (fluorescence recorded); and (iv) repeat to step ii for 50 cycles. Real-time data were collected by iQ5 optical system software v. 2.0 (Bio-Rad).

2.7. Immunocytochemistry

The antiserum was the same used in the ELISA analysis, which had been raised against the allatostatin BLAST-3 from *B. germanica* (Vilaplana et al., 1999). Staged late embryos, from day 10 to 17, were dissected, fixed in Bouin's fluid, embedded in paraffin and sectioned in 6-µm-thick sections (Bellés et al., 1994). The primary antiserum was used at a concentration of 1:400. The secondary antiserum was goat anti-rabbit conjugated to fluorescein isothiocyanate (FITC) (Sigma) at a concentration of 1:200. For control experiments, consecutive sections were incubated with primary antiserum and with the primary antiserum preabsorbed overnight at 4ºC with 100 nmol BLAST-3/ml (Maestro et al., 1998).

3. Results and discussion

3.1. Concentration pattern of JH III

JH III concentration was determined in *B. germanica* embryos during the whole embryogenesis period, which under our rearing conditions lasts 16 to 17 days (Maestro et al., 2005; Piulachs et al., 2010). JH III was the only JH homolog present in all samples, which is also the case in penultimate and last nymphal instars and in adult females of this cockroach (Treiblmayr et al., 2006). Indeed, JH III is the only JH homolog found in cockroaches (Baker et al., 1984). JH III was under the detection levels (<0.02 pmol) until day 7 after egg laying and ootheca formation (AOF), which represents 40% total development (TD), when the embryonic dorsal closure is produced (Tanaka, 1976). After that stage, JH III levels starts rising until a maximum of ca. 2 pmol/embryo on day 10 AOF (60% TD) (Fig. 1A). Then, JH III levels remain high until day 14 AOF (83% TD), and gradually decrease afterwards to undetectable levels the day before hatching (Fig. 1A). As far as molting hormone is concerned, ELISA-measured ecdysteroids (Maestro et al., 2005) indicated that there is a small peak on day 6 AOF (35% TD) and a much higher peak on day 13 (81% TD; Fig. 1A). Ultrastructural studies (Konopova & Zrzavy, 2005) have shown that *B. germanica* embryos deposit three sequential cuticles: EC1, EC2 and EC3. Morphological data indicate that EC2 and EC3 deposition, respectively, follows the two ecdysteroid peaks described above (Fig. 1A). Given that EC1 is produced between completion of the germ band and the beginning of appendage development, i.e. on day 3 AOF (20% TD), we presume that a further ecdysteroid pulse not detectable by ELISA measurements occurs around day 2 (see also, Piulachs et al., 2010). In this sense, previous data indicate that 2 days AOF *B. germanica* embryos show a transient but clear expression of the early ecdysteroid-induced gene E75A (Mané-Padrós et al., 2008), which supports the hypothesis of a cryptic ecdysteroid pulse on that day. Comparing the pattern of the two hormones, the

large peak of JH overlaps with the highest peak of ecdysteroids occurring on day 13 (81% TD; Fig. 1A), which gives rise to the EC3 deposition.

A similar pattern of JH concentration, comprising very low levels before dorsal closure, rising levels thereafter and decreasing levels before hatching has been reported in the cockroaches *N. cinerea* (Lanzrein et al., 1985) and *B. orientalis* (Short & Edwards, 1992). In *B. germanica*, if the highest levels are related to ootheca weight (39.0 ± 0.5 mg, n = 10, at 12 days AOF), they result in values around 550 ng JH III/g. These values are close to the maximal values found in *N. cinerea* and *B. orientalis*, and 20-fold the maximal values reported in *B. germanica* adult females (24.4 ng JH III/g in 7-day-old adult females, considering that the specimen weight without the ovaries is 87.4 ± 2.0 mg, n = 5) (Treiblmayr et al., 2006). In the embryo of *N. cinerea*, the maximal concentration of JH III was 10-fold higher than that found in the vitellogenic adult female (Lanzrein et al., 1985).

In *N. cinerea* (Lanzrein et al., 1984) and *D. punctata* (Stay et al., 2002), functional CA develop shortly after dorsal closure. Lanzrein and co-workers discovered that embryonic CA of *N. cinerea* are biosynthetically active, producing methyl farnesoate (MF) and JH III when incubated in vitro after dorsal closure (Lanzrein et al., 1984), and that these two products are synthesized de novo from acetate (Bürgin & Lanzrein, 1988). Embryos of *D. punctata* just after dorsal closure also produce MF and JH III, although JH III progressively predominates as development proceeds (Stay et al., 2002). *D. punctata* embryos show a peak of JH III release between 65% and 87% of development, and a posterior decrease (Holbrook et al., 1998), which coincides with the *B. germanica* embryo JH III titer profile (Fig. 1A). JH III and MF biosynthesis from CA at 40% post dorsal closure have been reported for cockroaches from different families (although not for *B. germanica*), and in most cases JH III biosynthetic rates measured

were more than 10-fold higher than those of MF (Li, 2007). It has been proposed that the accumulation of MF in embryos could be a consequence of the absence or inactivity of methyl farnesoate epoxidase combined with a limited oxygen supply and/or low rates of metabolism of MF, which suggests that this JH precursor may have not a specific function in the embryo (Lanzrein et al., 1984; Bürgin & Lanzrein, 1988; Stay et al., 2002).

3.2. AST concentrations and preproAST mRNA expression pattern

AST levels were quantified by ELISA in extracts of staged embryos. The pattern (Fig. 1B) showed that AST levels are low, although detectable (between 0.3 and 1 ng/embryo), until day 11 AOF (65% TD); then they steadily increase, reaching maximal values (around 7 ng/embryo) on day 15 AOF (89% TD), after which they remain approximately stable until the hatching day. Embryo AST levels had been previously reported in *D. punctata*, but they were estimated only in homogenates from young embryos before dorsal closure, and they increased from day 0 to day 2, and decreased thereafter (Woodhead et al., 2003).

PreproAST mRNA levels were estimated using RT-PCR + Southern-blot in staged embryos. The transcript was detected in very early embryogenesis (days 0-1, Fig. 1C), as well as in oocytes just before oviposition and in oothecae from virgin females (results not shown), therefore indicating that the transcript in this stage is from maternal origin. In *D. punctata,* AST peptide content in the eggs one day after oviposition nearly doubles the levels of the previous day (Woodhead et al., 2003), which suggests that preproAST mRNA supplied by the mother is actively translated. The same authors point to a possible function of AST peptides in the utilization of yolk in these very early

embryos (Woodhead et al., 2003). The pattern in *B. germanica* (Fig. 1C) shows that preproAST mRNA levels decrease after day 2 AOF (12% TD) and remain low until day 6 AOF (35% TD), and then steadily increase, reaching maximal values at around day 11 AOF (65% TD), which are maintained until hatching (Fig 1C). A similar pattern was found when preproAST mRNA levels were analyzed in oothecae of 0, 4, 8, 12 and 16 days using RT + real-time PCR (Fig. 3A). Results are expressed as copies of preproAST mRNA per thousand copies of actin5C mRNA. Although a maternal presence of preproAST mRNA was again suggested by this analysis, results from freshly formed oothecae (day 0) were not included in the figure because actin5c mRNA levels in this stage were three orders of magnitude lower than those of any other of the studied days (4, 8, 12 and 16 days), which makes inappropriate the comparison of day 0 values with those of other days. The high AST levels observed towards the end of embryogenesis, both in terms of peptide and mRNA (Fig. 1 B and C; Fig. 3A), coincide with the decrease of JH concentration observed during the same period (Fig. 1A), which suggests a cause-effect relationships.

3.3. Transcript levels of the methyl farnesoate epoxidase CYP15A1

Using degenerate primers and cDNA from 4- to 6-day-old adult female CA as template, a 917-bp fragment of a *B. germanica* homologue of CYP15A1 was obtained (GenBank accession number: FN826903). The cDNA sequence encoded for 305 amino acids fragment of the protein. The amino acid sequence showed a 72% of identity when compared with the corresponding fragment of *D. punctata* CYP15A1 (Helvig et al., 2004).

We used RT-PCR to analyze the tissue specificity of CYP15A1 expression. The occurrence of CY15A1 mRNA was examined in CA, muscle, fat body, ovary, colleterial gland and midgut tissues from 3- to 4-day-old females (Fig. 2), and it was detected only in the CA.

Furthermore, CYP15A1 expression was analyzed using RT + real-time PCR with the same samples as for the preproAST analysis (Fig. 3B). CYP15A1 transcripts were detected on day 4 AOF (25% TD), that is after the EC1 deposition and 3 days before dorsal closure; thereafter, the levels tended to increase but differences amongst the days studied were not statistically significant (Fig. 3B). Although JH was first detected on day 8 AOF (46% TD), we presume that CA primordium cells (Hartenstein, 2006) would be able to express CYP15A1 earlier in order to be prepared for the massive JH synthesis occurring in later stages; nevertheless, expression of CYP15A1 in embryonic cells other than those of CA cannot be ruled out. In *D. punctata*, embryonic CA acquire the ability to convert FA to JH in 28-30 day embryos (44-48% TD), thus suggesting that the production of the methyl farnesoate epoxidase is induced at this stage. Interestingly, farnesoic acid-*o*-methyltransferase appears in *D. punctata* CA early in embryonic life (by day 16, that is 25% TD), which allows the production of MF, but in the virtual absence of methyl farnesoate epoxidase, the final enzyme in the pathway, little JH is produced (Stay et al., 2002). The pattern obtained in *B. germanica* also indicates that CYP15A1 mRNA does not decrease at the end of embryogenesis (Fig. 3B), when JH titer falls down (Fig. 1A). This suggests that CYP15A1 mRNA expression does not modulate JH synthesis in *B. germanica*, at least in the embryo. In *D. punctata*, JH biosynthesis was significantly increased by incubating CA from embryos at different stages in medium containing farnesol (Holbrook et al., 1996),

which suggests again that JH biosynthesis must be regulated at early steps (Sutherland & Feyereisen, 1996).

3.4. Immunolocalisation of AST and final remarks

Towards the end of embryogenesis of *B. germanica* (day 11 AOF, 65% TD), AST immunorective material is detected in different areas of the central nervous system, like cells and nerves of the brain, suboesophageal ganglion and the other ganglia of the ventral nerve chord (Fig.4A and B), some of them unrelated to JH production. What is clear, however, is that immunostaining neatly labels the CA, where it forms a distinct network within the glands (Fig. 4A and B). In all cases immunostaining was abolished when the antiserum was preabsorbed overnight with the parent peptide (Fig. 4C and D) which assesses the specificity of the signal. In *D. punctata*, AST immunoreactivity has been well detected in brain lateral neurosecretory cell bodies and in their axon branches throughout the CA, in 26-day-old embryos (41% TD), that is before the JH synthesis started to rise (Stay et al., 2002). At this time, in vitro CA activity was not inhibited by AST treatment (Stay et al., 2002). CA started to be sensitive to AST inhibition just when they reached their maximal biosynthetic level, and became fully sensitive just before JH production started to decline (Stay et al., 2002). These results support the idea of ASTs as genuine inhibitors of JH production in cockroach CA at the end of embryogenesis.

In summary, the opposite patterns of JH and AST towards the end of embryogenesis in *B. germanica*, along with the detection of AST immunoreactivity in CA from late embryos, suggest that JH decline is caused by the increase of AST, which would inhibit JH production by the CA, although other factors, as an increase in JH

degradation rate, for example, might also contribute to reduce JH titer at this stage. Inhibition of JH production by ASTs in CA from late embryos incubated in vitro has been reported in *D. punctata* (Holbrook et al., 1996; Stay et al., 2002) which is in support of our hypothesis. In *B. germanica*, ascertaining whether ASTs inhibits JH synthesis in embryonic CA incubated in vitro would need further research. However, this kind of experiments would be technically difficult due to the small size of the embryo of *B. germanica* and to the fact that embryos are protected not only by the eggshell, but they are also tightly packaged within a hard ootheca.

Acknowledgments

This work was supported by the Spanish Ministry of Education and Science through the projects BFU2006-01090/BFI (to J.L.M.) and CGL2008-03517/BOS (to X.B.).

References

Aguilar, R., Maestro, J.L., Vilaplana, L., Pascual, N., Piulachs, M.D., Bellés, X., 2003. Allatostatin gene expression in brain and midgut, and activity of synthetic allatostatins on feeding-related processes in the cockroach *Blattella germanica*. Regul. Pept. 115, 171-177

Baker, F.C., Lanzrein, B., Miller, C.A., Tsai, L.W., Jamieson, G.C., Schooley, D.A., 1984. Detection of only JH III in several life-stages of *Nauphoeta cinerea* and *Thermobia domestica*. Life Sci. 35, 1553-1560

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. Regul. Pept. 53, 237-247

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

Bürgin, C., Lanzrein, B., 1988. Stage-dependent biosynthesis of methyl farnesoate and juvenile hormone III and metabolism of juvenile hormone III in embryos of the cockroach, *Nauphoeta cinerea*. Insect Biochem. 18, 3-9

Erezyilmaz, D.F., Riddiford, L.M., Truman, J.W., 2004. Juvenile hormone acts at embryonic molts and induces the nymphal cuticle in the direct-developing cricket. Dev. Genes Evol. 214, 313-323

Hartenstein, V., 2006. The neuroendocrine system of invertebrates: a developmental and evolutionary perspective. J. Endocrinol. 190, 555-570

Helvig, C., Koener, J.F., Unnithan, G.C., Feyereisen, R., 2004. CYP15A1, the cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach corpora allata. Proc. Natl. Acad. Sci U S A 101, 4024-4029

Holbrook, G.L., Chiang, A.-S., Schal, C., 1996. Allatostatin Inhibition and Farnesol Stimulation of Corpus Allatum Activity in Embryos of the Viviparous Cockroach, *Diploptera punctata*. Arch. Insect Biochem. Physiol. 32, 341-352

Holbrook, G.L., Chiang, A.-S., Lee, Y.-J., Lin, C.-Y., Schal, C., 1998. Juvenile hormone synthesis in relation to corpus allatum development in embryos of the viviparous cockroach *Diploptera punctata*. Invert. Reprod. Develop. 33, 69-79

Konopova, B., Zrzavy, J., 2005. Ultrastructure, development, and homology of insect embryonic cuticles. J. Morphol. 264, 339-362

Lanzrein, B., Imboden, H., Bürgin, C., Brüning, E., Gfeller, H., 1984. On titers, origin, and functions of juvenile hormone III, methylfarnesoate, and ecdysteroids in embryonic development ot the ovoviviparous cockroach *Nauphoeta cinerea*. In: Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones, Hoffmann, J., Porchet, M. (Eds.), Springer-Verlag, Heidelberg, pp 454-465

Lanzrein, B., Gentinetta, V., Abegglen, H., Baker, F.C., Miller, C.A., Schooley, D.A., 1985. Titers of ecdysone, 20-hydroxyecdysone and juvenile hormone III throughout the life cycle of a hemimetabolous insect, the ovoviviparous cockroach *Nauphoeta cinerea*. Experientia 41, 913-917

Li, X., 2007. Juvenile hormone and methyl farnesoate production in cockroach embryos in relation to dorsal closure and the reproductive modes of different species of cockroaches. Arch. Insect Biochem. Physiol. 66, 159-168

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*. Arch. Insect Biochem. Physiol. 37, 269-282

Maestro, J.L., Bellés, X., 2006. Silencing allatostatin expression using double-stranded RNA targeted to preproallatostatin mRNA in the German cockroach. Arch. Insect Biochem. Physiol. 62, 73-79

Maestro, J.L., Cobo, J., Bellés, X., 2009. Target of rapamycin (TOR) mediates the transduction of nutritional signals into juvenile hormone production. J. Biol. Chem. 284, 5506-5513

Maestro, O., Cruz, J., Pascual, N., Martín, D., Bellés, X., 2005. Differential expression of two RXR/ultraspiracle isoforms during the life cycle of the hemimetabolous insect *Blattella germanica* (Dictyoptera, Blattellidae). Mol. Cell. Endocrinol. 238, 27-37

Mané-Padrós, D., Cruz, J., Vilaplana, L., Pascual, N., Bellés, X., Martín, D., 2008. The nuclear hormone receptor BgE75 links molting and developmental progression in the direct-developing insect *Blattella germanica*. Dev. Biol. 315, 147-160

Piulachs, M.D., Pagone, V., Bellés, X., 2010. Key roles of the *Broad-Complex* gene in insect embryogenesis. Insect Biochem. Mol. Biol. 40, 468-475

Rembold, H., Lackner, B., 1985. Convenient method for determination of picomole amounts of juvenile hormone. J. Chromatogr. 323, 355-361

Short, J.E., Edwards, J.P., 1992. Levels of juvenile hormone III during embryonic development in the oriental cockroach *Blatta orientalis* (L.) (Dictyoptera, Blattidae). In: Insect Juvenile Hormone Research, Mauchamp, B., Couillaud, F., Baehr, J.C. (Eds.), INRA, Paris, pp 19-25

Stay, B., 2000. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. Insect Biochem. Mol. Biol. 30, 653-662

Stay, B., Zhang, J.R., Tobe, S.S., 2002. Methyl farnesoate and juvenile hormone production in embryos of *Diploptera punctata* in relation to innervation of corpora allata and their sensitivity to allatostatin. Peptides 23, 1981-1990

Stay, B., Tobe, S.S., 2007. The role of allatostatins in juvenile hormone synthesis in insects and crustaceans. Annu. Rev. Entomol. 52, 277-299

Sutherland, T.D., Feyereisen, R., 1996. Target of cockroach allatostatin in the pathway of juvenile hormone biosynthesis. Mol Cell Endocrinol 120, 115-123

Tanaka, A., 1976. Stages in the Embryonic Development of the German Cockroach, *Blattella germanica* LINNÉ (Blattaria, Blattellidae). Kontyû, Tokyo 44, 512-525

Treiblmayr, K., Pascual, N., Piulachs, M.D., Keller, T., Belles, X., 2006. Juvenile hormone titer versus juvenile hormone synthesis in female nymphs and adults of the German cockroach, *Blattella germanica*. 7pp. J. Insect Science 6:47, available online: insectscience.org/6.47

Vilaplana, L., Maestro, J.L., Piulachs, M.D., Bellés, X., 1999. Determination of allatostatin levels in relation to the gonadotropic cycle in the females of *Blattella germanica* (L.) (Dictyoptera, Blattellidae). Physiol. Entomol. 24, 213-219

Woodhead, A.P., Thompson, M.E., Chan, K.K., Stay, B., 2003. Allatostatin in ovaries, oviducts, and young embryos in the cockroach *Diploptera punctata*. J. Insect Physiol. 49, 1103-1114

FIGURE LEGENDS

Fig. 1. A. Juvenile hormone III (JH III) levels in embryos of *Blattella germanica*. Values represent the mean ± SEM; the number of replicates is indicated at the top of each bar; the asterisk indicates values under level detection (<0.02 pmol); nd: not determined; different letters indicate statistical differences based on one-way ANOVA Tukey's multiple comparison test; the arrows indicate the peaks of ecdysteroids and the deposition of the three embryonic cuticles (EC1-3) (Konopova & Zrzavy, 2005; Maestro et al., 2005; Mané-Padrós et al., 2008; Piulachs et al., 2010); dorsal closure and hatching events are also indicated. B. AST contents expressed as the mean ± SEM of BLAST-3 equivalents; the number of replicates is indicated at the top of each bar; different letters indicate statistical differences based on one-way ANOVA Tukey's multiple comparison test (except for day 2 which was not included in the test because it corresponds to a single measurement). C. Southern-blots showing preproallatostatin (PreproAST) and actin5C mRNA levels; the blots are representative of 4 replicates.

Fig. 2. Expression of CYP15A1 in different tissues of *Blattella germanica*. The expression of CYP15A1 was measured in CA, muscle, fat body, ovary, colleterial gland and midgut tissues of 3- to 4-day-old *B. germanica* adult females. Actin5C expression levels were used as a reference.

Fig. 3. Expression patterns of preproallatostatin (preproAST) and CYP15A1 in embryos of *Blattella germanica*. Results show mRNA levels of preproAST (A) and CYP15A1 (B) for embryos of 4, 8, 12 and 16 days after ootheca formation (AOF) quantified by real-time PCR. Y-axis indicate copies of the studied transcript per a thousand copies of

actin5C. Results are expressed as the mean ± SEM (n = 3). Different letters at the top of the bars indicate statistical differences based on one-way ANOVA Tukey's multiple comparison test.

Fig. 4. Paraffin sections of *Blattella germanica* embryos immunostained with a BLAST-3 antiserum and revealed with a secondary antibody conjugated to FITC. A, B. Sagital sections of the anterior body region of an 11 days after ootheca formation (AOF) embryo; arrowheads indicate immunopositive staining. C. Sagital section of the mesothoracic ganglion of a 15 days AOF embryo showing positive immunostaining. D. Section consecutive to C using primary antiserum preabsorbed with the parent peptide, showing no immunostaining, which assesses the specificity of the signal. Br: brain; CA: corpora allata; SOG, suboesophageal ganglion; T1: prothoracic ganglion; T2: mesothoracic ganglion.

Figure 1

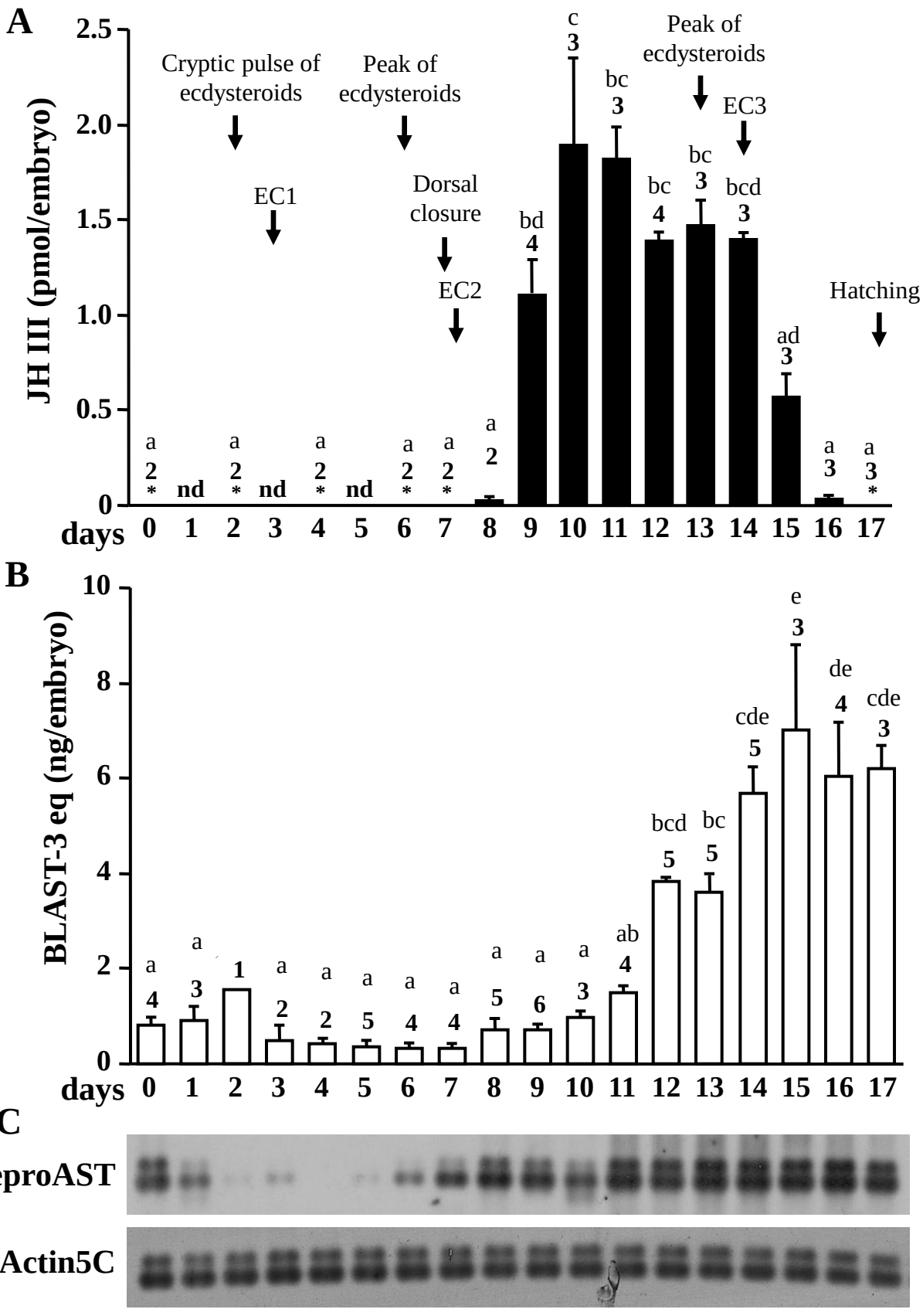
A
JH III (pmol/embryo)
2.5
2.0
1.5
1.0
0.5
0
Cryptic pulse of ecdysteroids
Peak of ecdysteroids
EC1
Dorsal closure
EC2
Peak of ecdysteroids
EC3
Hatching
days 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
B
BLAST-3 eq (ng/embryo)
10
8
6
4
2
0
days 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
C
PreproAST
Actin5C

Figure 2

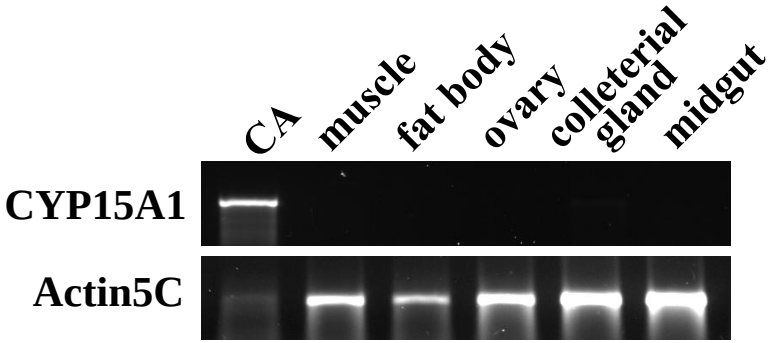
CA
muscle
fat body
ovary
colleterial gland
midgut
CYP15A1
Actin5C

Figure 3

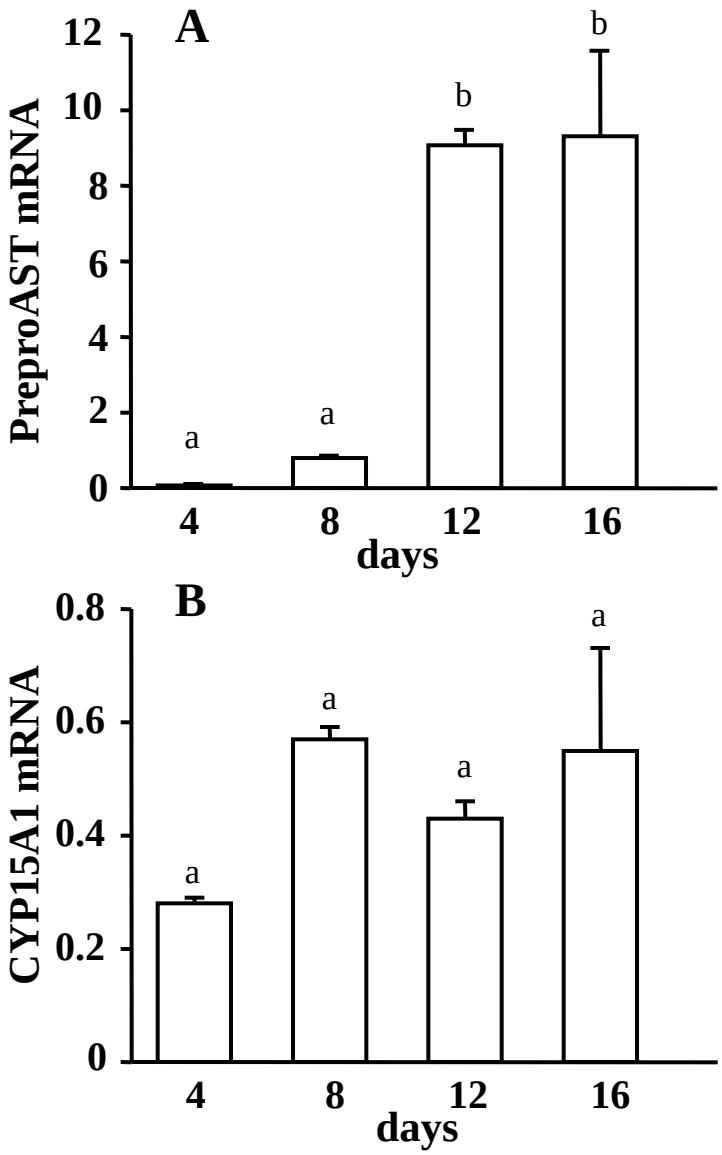
A
PreproAST mRNA
12
10
8
6
4
2
0
a
a
b
b
4
8
12
16
days
B
CYP15A1 mRNA
0.8
0.6
0.4
0.2
0
a
a
a
a
4
8
12
16
days

Figure 4 (colour version)