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Reconstruction of ancestral FGLamide-type insect allatostatins: A novel approach to the study of allatostatin function and evolution

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ABSTRACT

Allatostatins (ASTs) are a class of regulatory neuropeptides, with diverse functions, found in an array of invertebrate phyla. ASTs have complex gene structure, in which individual ASTs are cleaved from a precursor peptide. Little is known about the molecular evolution of AST structure and function, even in extensively studied groups such as cockroaches. This paper presents the application of a novel technique for the analysis of this system, that of ancestral reconstruction, whereby ancestral amino acid sequences are resurrected in the laboratory. We inferred the ancestral sequences of a well-characterized peptide, AST 7, for the insect ancestor, as well as several cockroach ancestors. Peptides were assayed for *in vitro* inhibition of JH production in *Diploptera punctata* and *Periplaneta americana*. Our results surprisingly, indicate a decrease in potency of the ancestral cockroach AST7 peptide in comparison with more ancient ones such as the ancestral insect peptide, as well as more recently evolved cockroach peptides. We propose that this unexpected decrease in peptide potency at the cockroach ancestor may be related to the concurrent increase in peptide copy number in the lineages leading to cockroaches. This model is consistent with current physiological data, and may be linked to the increased role of ASTs in the regulation of reproductive processes in the cockroaches.

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1. Introduction

Allatostatins (ASTs) are a class of regulatory neuropeptides found in a diversity of invertebrate phyla. The cockroach type AST, or FGLamide (FGLa) types, were first discovered in the viviparous cockroach *Diploptera punctata* (Woodhead et al., 1989; Pratt et al., 1989). FGLa ASTs share the C-terminal motif (Y/F)XFG(L/I)-NH₂ which forms the core active region of the peptide (Tobe and Bendena, 2006). However, not all AST-like peptides possess the same C-terminal sequence; for example, nematode sequences terminate in MGL/FGF/MGF (Nathoo et al., 2001; Husson et al., 2005). Whereas ASTs were named and characterized as a consequence of their ability to inhibit juvenile hormone (JH) biosynthesis by the corpora allata (CA), these peptides have also been found to serve many other functions. ASTs act as myomodulators across a wide variety of invertebrate phyla including helminths, Crustacea, and the insect orders Diptera, Lepidoptera, Dictyoptera and Orthoptera (for example see Jorge-Rivera and Marder, 1997; Mousley et al., 2005; Bendena et al., 2008; Lange et al., 1995; Aguilar et al., 2003; Predel et al., 2001; Duve et al., 1995, 1996; Veelaert et al., 1996; Vanden Broeck et al., 1996). The FGLa ASTs also inhibit vitellogenin production in the fat body of the

German cockroach *Blattella germanica*, stimulate the activity of carbohydrate-metabolizing enzymes in the midgut of *D. punctata* and inhibit cardiac activity in *B. germanica* (Martin et al., 1996; Fusé et al., 1999; Vilaplana et al., 1999).

From a molecular evolutionary standpoint, ASTs are fascinating as a consequence of their complex gene structure, and striking diversity of function. ASTs arise from preproallatostatin, the precursor peptide, in which multiple ASTs are cleaved at dibasic KR/RR/KK/RK endoproteolytic cleavage sites (Tobe and Bendena, 2006). These repeats are assumed to be the result of duplication events, and both the amino acid sequence of the precursor peptide and its structural organization can vary greatly across extant species (Bellés et al., 1999; Bendena et al., 1999). In terms of molecular evolution, this presents an interesting situation where peptide sequences within a gene can diverge and acquire new functions over time, yet are regulated together as part of the same precursor.

The evolutionary history of ASTs and their diversity of function are of particular interest in insects. Many studies have been conducted in insects but the functional differences in AST peptides with respect to phylogeny remain poorly understood. Although the myomodulatory role of ASTs appears to be conserved across invertebrate groups, this is not the case for other AST functions. Physiological studies examining the effect of ASTs on JH production by insect corpora allata have been performed in many species. Although FGLa ASTs are present in

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both hemimetabolous and holometabolous insects, the ability to regulate JH biosynthesis appears to be limited to only some of the hemimetabolous insect orders—Orthoptera, Dictyoptera and Isoptera (for a review see Stay and Tobe, 2007). Thus far, other functions of ASTs, such as the aforementioned regulation of digestive enzyme activity, have also only been described in the Hemimetabola, and in particular only in the Dictyoptera (Martin et al., 1996; Fusé et al., 1999; Vilaplana et al., 1999).

Even within the Hemimetabola, the relationship between primary structure of a peptide and its function remains unclear. Although Bellés et al. (1999) demonstrated that in cockroaches AST peptides are highly conserved in terms of sequence, the potency of a given peptide is not necessarily conserved across species. The seventh AST (AST 7) of the cockroaches *B. germanica* and *D. punctata* both inhibit JH biosynthesis but with different potencies; AST 7 is more potent in *D. punctata* than in *B. germanica*, by several orders of magnitude (Bellés et al., 1994; Tobe et al., 2000). ASTs do not necessarily serve the same functions in all closely related species; ASTs inhibit JH biosynthesis in the cricket *Gryllus bimaculatus* but have no effect in another Orthopteran, the locust *Schistocerca gregaria* (Lorenz et al., 1995, 1999; Veelaert et al., 1996). Molecular studies examining the relationships between individual ASTs and among AST precursor genes have attempted to address questions regarding function and phylogeny (Bellés et al., 1999; Bendena et al., 1999). However, there are still no conclusive answers regarding the origin and evolutionary history of AST function in Hemimetabola or any other group.

Among insects, cockroaches are of particular interest because of their diversity of reproductive modes. In cockroaches, three basic modes of reproduction occur, oviparity in which fertilized eggs develop outside of the body, ovoviviparity in which fertilized eggs are carried in a brood sac, and viviparity in which fertilized eggs are carried within the brood sac and are nurtured by the female (Roth, 1970). Reproductive processes such as vitellogenesis and oocyte maturation are linked to the well-coordinated regulation of JH production by the corpora allata during the reproductive cycle (Tobe and Stay, 1977). This requirement for the regulation of JH production is likely to be linked to the increased number of functional roles and potency of ASTs in cockroaches. Additionally, AST peptides have also been well studied in the Dictyoptera and much comparative data are available. The cockroach AST peptides have been well characterized in terms of regulation of JH production in the greatest number of hemimetabolous species, and thus lend themselves well to further study (Tobe et al., 2000; Bellés et al., 1994; Weaver et al., 1994; Lorenz et al., 1999; Yagi et al., 2005).

In recent years, the scope of species in which ASTs have been identified has grown considerably. FGLa immunoreactivity has been described in many lower invertebrates such as Trematodes and Hydrozoa, as well as in Gastropoda and Cephalopoda, but no AST-like sequences have been identified from any of these groups to date (Bendena et al., 1999; Smart et al., 1994). The available AST sequence data have also expanded. In addition to insects, FGLa AST precursors have been described in several crustacean genera and neuropeptide-like-protein encoding sequences with sequences similar to FGLa have been identified in nematodes via genome project searches (Duve et al., 1997a; Billimoria et al., 2005; Huybrechts et al., 2003; Dirksen et al., 1999; Yin et al., 2006; Duve et al., 2002; Yasuda-Kamatani and Yasuda, 2006; Nathoo et al., 2001). This expansion in sequence knowledge has enabled more sophisticated molecular evolutionary studies, including the application of ancestral reconstruction techniques to the analysis of AST function.

Experimental ancestral reconstruction approaches use phylogenetic statistical methods of sequence analysis to infer sequences

that existed in the past; these inferred sequences are then synthesized in the laboratory and studied in functional assays (Thornton, 2004). Ancestral reconstruction methods can provide information about the evolutionary history of functional and biochemical characteristics of proteins and peptides which could not otherwise be experimentally studied (Chang and Donoghue, 2000). These methods have been successful in previous studies experimentally reconstructing chymases, visual pigments and hormone receptors, among others, as well as for studying the paleobiology of extinct species (Chandrasekharan et al., 1996; Chang et al., 1995, 2002; Thornton et al., 2003; Gaucher et al., 2008). However, ancestral reconstruction methods have never been before applied to ASTs or any other complex family of invertebrate peptide hormones.

Genes such as those coding for the FGLa ASTs present special challenges to ancestral reconstruction methodologies as a consequence of peptide shuffling and the expansion and contraction of peptide number. Here, we present a reconstruction of cockroach full-length AST precursor genes and a reconstruction of highly conserved peptides from insect ASTs for ancestral nodes within cockroach lineages, as well as the insect ancestor. Problems with positional homology of AST peptides prevented the inclusion in our analyses of the crustacean sequences; instead a frequency analysis of ASTs in arthropod groups was used to understand the pattern of AST occurrence in different taxa. To examine the role of amino acid changes in terms of AST activity, we have performed assays of ancestral peptide AST 7 at reconstructed nodes to show their potency in inhibition of JH biosynthesis *in vitro*.

2. Materials and methods

2.1. Ancestral reconstruction

To reconstruct the ancestral FGLa AST precursor gene, we first constructed an alignment of all currently known hemimetabolous insect AST precursor gene sequences. FGLa AST precursor genes of six cockroaches, and two Orthopteran insects were used (GenBank/EMBL accession nos. *D. punctata* U00444, *Periplaneta americana* X91029, *B. germanica* AF068061, *Blaberus craniifer* F068062, *Supella longipalpa* AF068063, *Blatta orientalis* AF068064, *G. bimaculatus* AJ302036, *S. gregaria* Z58819). The sequences were translated to amino acids, aligned using ClustalX 2.0 (Thompson et al., 1997) and adjusted by eye to ensure that known structure/functional motifs in the active peptide domains of the AST gene were in alignment. The amino acid alignment (termed 'hemimetabolous alignment') is presented in Fig. S1. Although several holometabolous insect AST precursor genes are known, they were found to be too divergent from the hemimetabolous insect sequences to align reliably across the whole gene. We therefore constructed a second, shorter alignment (termed 'insect alignment', Fig. 3) that comprised only of relatively conserved regions of several holometabolous and hemimetabolous insect AST genes. Identical sequences within a family were removed to allow more rapid analysis (species names and GenBank/EMBL accession nos. *D. punctata* U00444, *P. americana* X91029, *B. germanica* AF068061, *B. craniifer* AF068062, *G. bimaculatus* AJ302036, *S. gregaria* Z58819, *Spodoptera frugiperda* AJ555184, *Helicoverpa armigera* AF015296, *Bombyx mori* NM_001043571, *Drosophila melanogaster* AF263923, *Drosophila grimshawi* (see Bowser and Tobe, 2007), *Apis mellifera* XM_001120780, *Calliphora vomitoria* (see East et al., 1996), *Aedes aegypti* U66841, *Anopheles gambiae* XM_313511, *Calanus finmarchicus* EU000307).

Ancestral reconstructions were carried out using both the codeml (for amino acid and codon data) and baseml

(for nucleotide data) programs of the PAML software package, version 3.15 (Yang, 1997), and the rje_ancseq module of the GASP software package (Edwards and Shields, 2004). Maximum likelihood/Bayesian ancestral reconstruction methods infer the most likely ancestral sequence reconstructions for nodes on a given phylogeny, according to a specified model of evolution. Most probable ancestral character states are inferred for all sites in the alignment, for any internal node (Yang et al., 1995; Yang, 2006). While a variety of substitution models can be considered in codeml and baseml, the methods these programs implement either ignore gaps or treat gaps as ambiguous character states. The module rje_ancseq, while not as accurate at inferring ancestral states as codeml, explicitly considers the historical pattern of insertions and deletions in the gene of interest, using parsimony to assign gaps prior to sequence reconstruction (Edwards and Shields, 2004). Since the hemimetabolous alignment contained many gaps (Fig. S1), we chose to combine both approaches, and employed the rje_ancseq program to infer the ancestral gap pattern, and both rje_ancseq and codeml/baseml to infer the ancestral sequence data. The shorter insect alignment, which contained only areas of relatively high sequence similarity, was not analyzed using rje_ancseq as it contained relatively few gaps.

The JTT (Jones et al., 1992) amino acid substitution matrix was used to infer the ancestral sequence data in rje_ancseq, while the JTT (Jones et al., 1992) and WAG (Whelan and Goldman, 2001) amino acid substitution models, the M0 and M3 (Goldman and Yang, 1994; Yang et al., 2000) codon substitution models, and the HKY (Hasegawa et al., 1985) nucleotide substitution model, were used to estimate ancestral sequence data in codeml/baseml. The analyses carried out in codeml/baseml were performed both with and without the addition of a gamma parameter, which allows the overall substitution rate in the maximum likelihood analysis to vary across sites (Yang, 1996). In all cases, the addition of the gamma parameter led to a significantly better fitting model (P -values < 0.001) as determined by likelihood ratio tests (Felsenstein, 1981; Yang, 2006), so only those results are presented (see Table S3). Trees reflecting current understanding of insect phylogenetics (Figs. 1 and 2; Kambhampati, 1995, 1996) (Fig. 3; Wheeler et al., 2001; Kristensen, 1991; Pashley et al., 1993; Boudreaux, 1979; Regier et al., 2005) were used in the ancestral reconstruction analyses. The ancestral sequence data, along with posterior probability estimates for each amino acid at each site, were extracted from the codeml/baseml output files using a customized Perl script.

2.2. Sequences collection and database analysis

A sequence database of all known FGLa allatostatin precursors and peptide sequences was collected from literature, GenBank and EMBL (Table S1). This database was similar to AST data from Liu et al. (2006); however, our dataset includes peptides isolated by protein methods as well (<http://signalling.peptides.be>). Two nematode species (*Caenorhabditis elegans* and *Caenorhabditis briggsae*) are listed but were not used for analysis. The compiled list was entered into a Microsoft access database and searched using a visual basic executable. Each entry was tagged with one of the following classifications: Crustacea, Holometabola or Hemimetabola. Using search parameters, the number of total sequences within each groups were counted and the number ASTs shared between groups was determined (Fig. 4). A degenerate search was used to determine the frequency of AST sequences within the dataset. Sequences of interest were input into the search executable and all sequences identical to the input sequence or containing the input sequence were called up (i.e., zero amino

acid differences). In degenerate searches, either one or two amino acid sites were allowed to differ from the input sequence, all resulting hits containing the sequence were counted.

2.3. Radiochemical assays of JH release in vitro

2.3.1. Animals

Assays were conducted on *D. punctata* and *P. americana*. *D. punctata* were kept at 27 °C in constant darkness and given lab chow and water *ad libitum*. Newly ecdysed mated adult female *D. punctata* were selected, removed from the colony, placed in containers and provided food and water for 7 days at which point the animals were dissected. *P. americana* were kept at 27 °C on a 12:12 light:dark cycle with lab chow and water available *ad libitum*. Last instar females were generously provided by the animal physiology teaching labs at the Department of Cell and Systems Biology at the University of Toronto (Toronto, Canada). Newly molted adult females were isolated from this group and dissected on day 4.

2.3.2. Peptides

Peptides predicted by ancestral reconstruction were synthesized by GL Biochem Ltd. (Shanghai, China) at >95% purity. The reconstruction of the ancestral cockroach AST 7 predicted at nodes 12 and 14 were synthesized—the Blattidae ancestor (Ba) SPSGMQRLYGFGL-NH₂ and the cockroach ancestor (Ca) APSGMQRLYGFGL-NH₂, respectively (Fig. 1). For the reconstruction of conserved regions of both hemimetabolous and holometabolous insects, AST 7 from nodes 1 and 3 were synthesized—the insect ancestor (Ia) SRLYSFGL-NH₂ and the cockroach ancestor, an N-terminally truncated version of Ca (Ca-truncated) QRLYGFGL-NH₂, respectively (Fig. 3). All peptide stocks were prepared in ddH₂O.

2.3.3. Radiochemical assay in vitro

Day 7 mated adult female *D. punctata* and virgin day 4 *P. americana* were immobilized on ice and corpora allata dissected under sterile conditions. Only oviposited *D. punctata* females were used, to ensure uniform staging. A short-term *in vitro* assay of JH release in T199 medium (GIBCO) [2% Ficoll, 1.3 mM CaCl₂ · 2H₂O and 3 μCi/ml L-[methyl-¹⁴C]methionine (2.07 GBq/mmol; Amersham)] followed by rapid partition was conducted on individual corpora allata according to Feyereisen and Tobe (1981) and Tobe and Clarke (1985). Dose–response curves were generated using the percent inhibition of JH release and analyzed using non-linear regression.

3. Results

3.1. Ancestral reconstruction

For both the cockroach and insect datasets, ancestral sequences were estimated using a variety of codon, nucleotide and amino acid-based likelihood models of substitution (Yang, 1997). Since these methods do not explicitly consider gaps in the alignment, where necessary, we also estimated ancestral sequences using a method that does consider gaps (Edwards and Shields, 2004). A reduced alignment consisting only of highly conserved regions of the AST precursor gene was also analyzed, and this truncated alignment contained considerably fewer gaps. Within datasets, the ancestral reconstruction results are generally similar regardless of the method or substitution model used. Likelihood/Bayesian methods of ancestral reconstruction include posterior probability values, an indication of the reliability of the

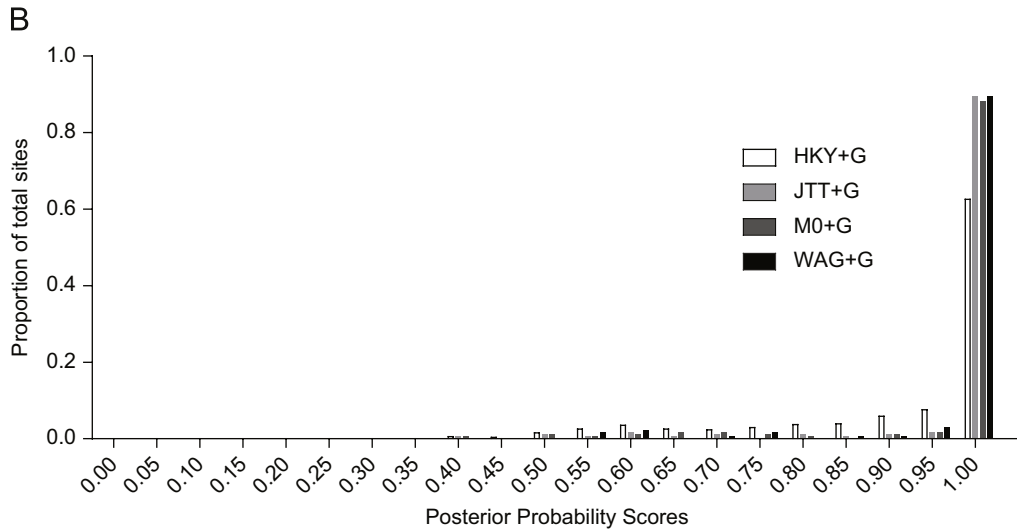
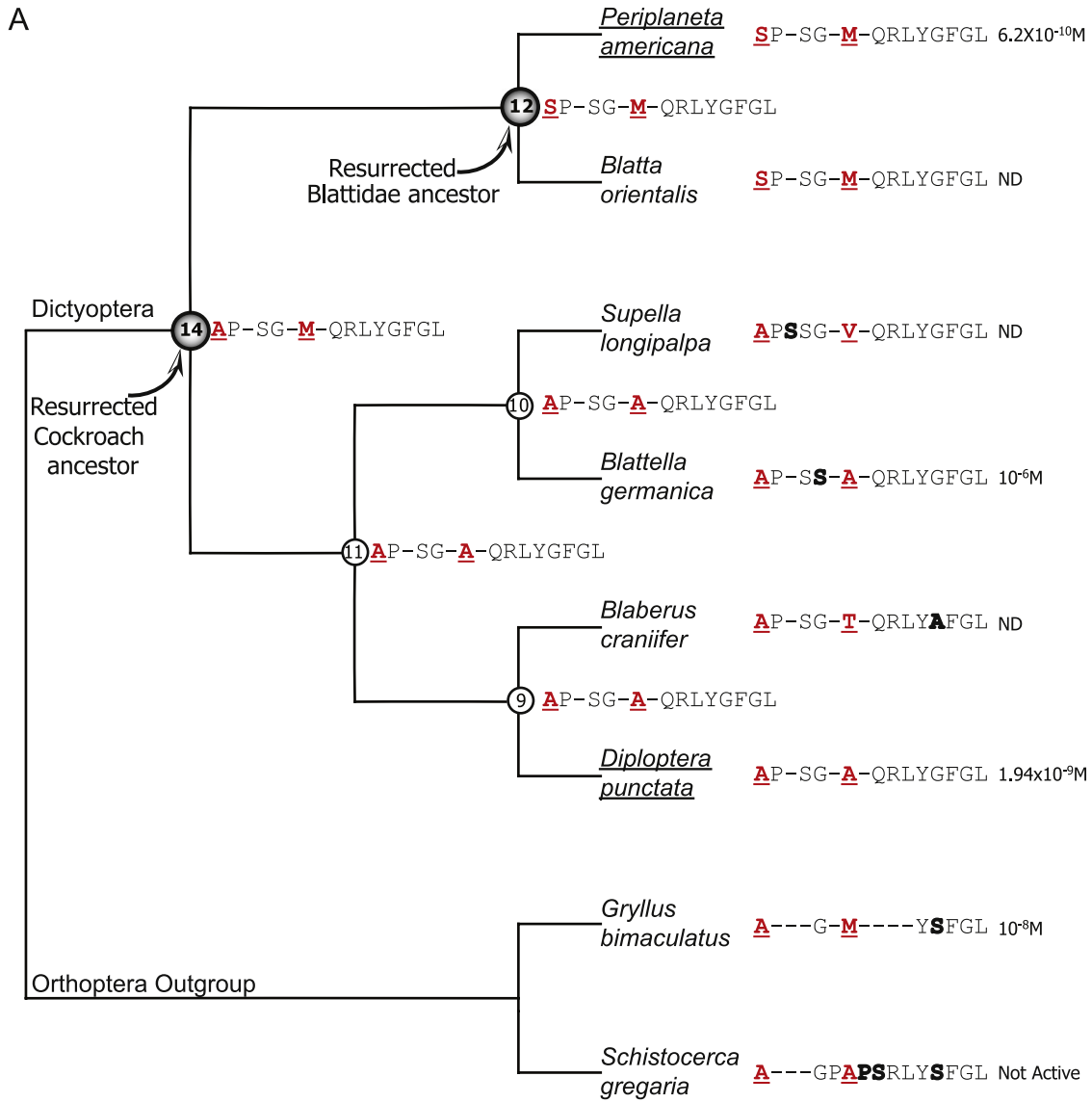


Fig. 1. Ancestral reconstruction of Dictyopteran ASTs. (A) Phylogeny of hemimetabolous insect groups used in our analysis. Shaded circles indicate the nodes where an AST peptide was experimentally resurrected. Species where ASTs were tested are underlined. Mapped on to this phylogeny are the inferred ancestral amino acid sequences of AST 7, underlined red letters show changes which occur across all nodes and bold letter show amino acids that change across extant taxa. The EC₅₀ values for AST 7 inhibition of JH release in the extant peptides are shown on the right, ND indicates a species for which no data are available (Weaver et al., 1994; Bellés et al., 1994; Tobe et al., 2000; Lorenz et al., 1999). (B) Distribution of posterior probabilities across sites at node 14 for different models of ancestral reconstruction as implemented in PAML. Most sites show probabilities >0.95, indicating that the amino acid reconstructions are likely to be correct. Sites inferred under the nucleotide model HKY+G have relatively lower probabilities due to third position variability in codons.

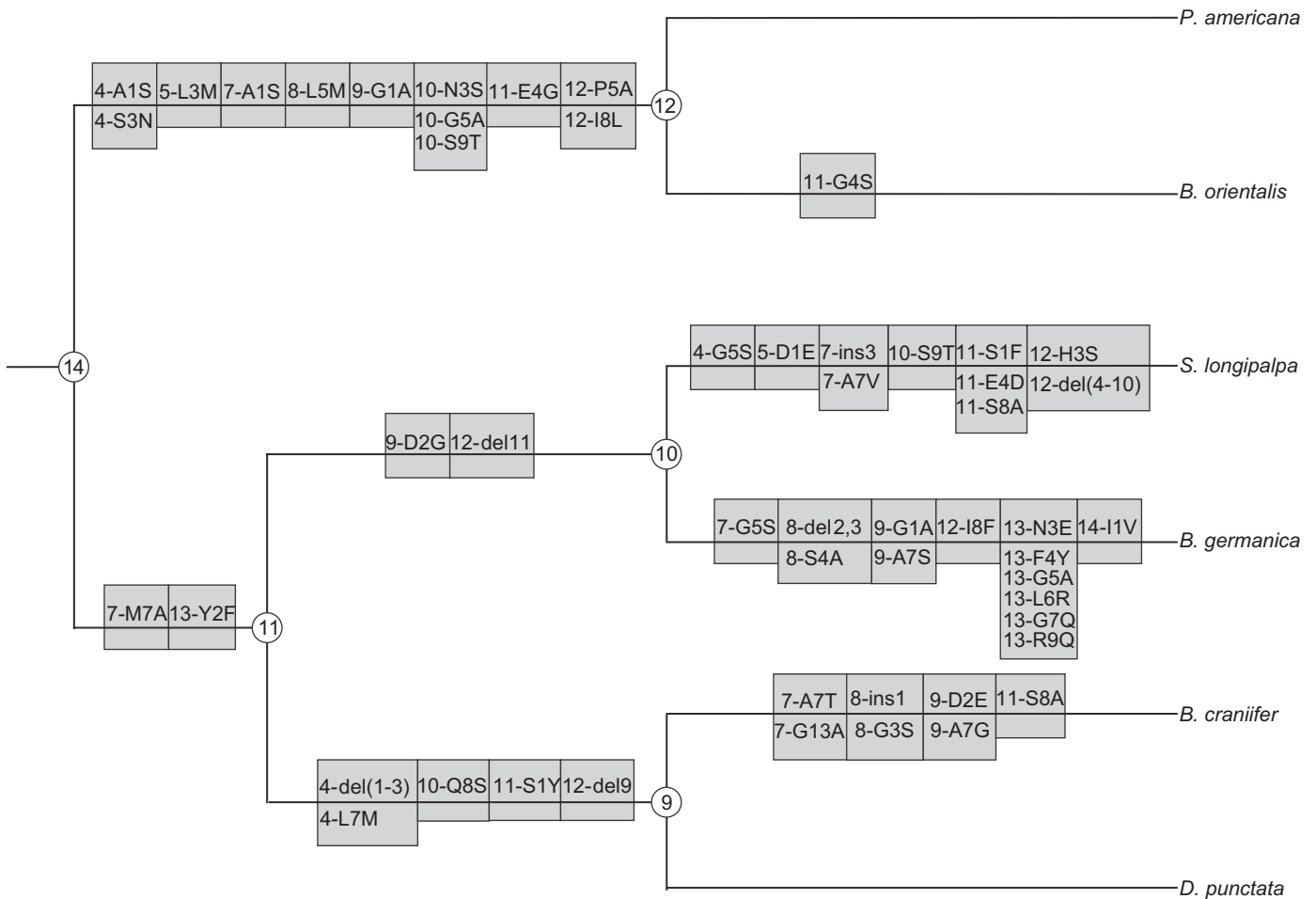


Fig. 2. Map of amino acid changes across cockroach nodes for AST peptides inferred in the reconstructed precursor genes. Each box represents an AST peptide, the first number within the box denotes the AST followed by the amino acid and N-terminal site number at which the change occurs. For example, 7-M5A represents a change in the fifth site of AST 7 from methionine to alanine. Deletions and insertions are shown using the abbreviations del and ins, respectively. For complete alignment, see supplementary data (Fig. S1).

reconstruction given a specific substitution model. These posterior probabilities, as calculated in PAML, were generally high across the different methods, particularly for the nodes reconstructed (Figs. 1B and 3C; Table S2). Ancestral reconstructions for the different models are contained in the supplementary data (Figs. S1 and S2).

For the hemimetabolous dataset, the inferred amino acid changes across the phylogeny are shown in Figs. 1 and 2. In particular, the reconstruction of AST 7 showed several interesting amino acid substitutions. The first substitution occurred along the branch connecting nodes 14 (the parent node, representing the ancestor of all cockroaches) and node 12, and involved substituting a non-polar alanine for a polar serine residue. The second substitution occurred along the branch-connecting node 14 (again, the node representing the ancestor of all cockroaches) and node 11, and involved substituting a methionine for an alanine. In the 'insect' dataset, we also noted two interesting substitutions in AST 7 (Fig. 3B). Both of these substitutions appear to have arisen at node 3 (representing the ancestor of the cockroaches). The first substitution involved substituting a serine for a glutamine (both of which are polar, non-charged residues), whereas the second involved substituting a polar serine for a non-polar glycine. Interestingly, the maximum likelihood ancestral sequence of AST 7 was identical at the nodes representing the ancestor of the hemimetabolous insects and the ancestor of all insects. Several reconstructed peptides, present at nodes where

changes in peptide copy number are thought to have occurred, were chosen for further analysis. Peptides were selected based on the presence of amino acid substitutions and the availability of comparative physiological data. Therefore, the inferred AST 7 peptides from node 14 and 12 from the cockroach dataset, and nodes 1 and 3 from the insect dataset, were synthesized and assayed for biological activity (Figs. 1A and 3A).

3.2. Sequences and database analysis

The collection of sequence data for all known FGLa ASTs revealed a great number of ASTs. In total, cockroach type AST-like sequences have been reported in 43 species; 10 Hemimetabola, 23 Holometabola, 8 Crustacea and 2 Nematoda. AST sequences have been obtained using peptide isolation and identification as well as molecular methods so not all species have known precursor genes. Of the 43 species listed, genetic sequence information is known for only 31.

Within the Arthropoda, a total of 431 FGLa AST sequences have been identified, and 233 different sequences and of those, 168 are specific to a species. Little overlap occurs between the ASTs found in Insecta and Crustacea. The Hemimetabola share four sequences with the Crustacea and only two with the Holometabola (Fig. 4A). Because a lack of positional homology restricted our reconstruction only to the conserved regions of insect precursor genes, a search strategy was implemented to analyze peptide patterns

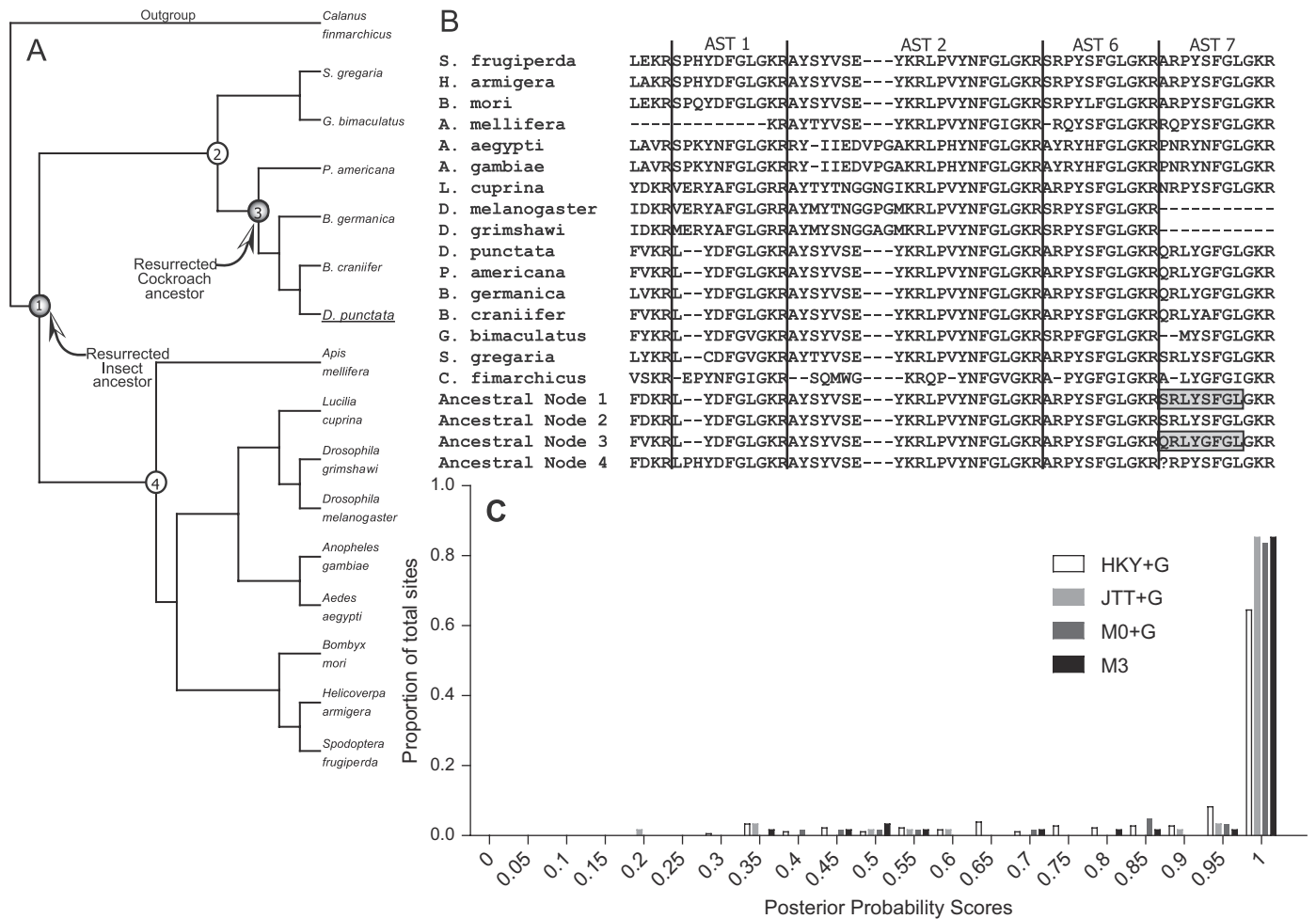


Fig. 3. Ancestral reconstruction of ancient insect ASTs. (A) Insect phylogeny of insect orders used in our analysis. The nodes where an AST was resurrected are indicated by arrows and shaded circles. All peptides were tested in *D. punctata*, which is shown in underlined text in the phylogeny. (B) Alignment of conserved insect ASTs created using ClustalX and modified by eye. Ancestral sequences for each node reflect a consensus of all models used for reconstruction as reported in Fig. S2 and resurrected peptides are indicated with a gray box. Variable sites are indicated by a question mark (?). (C) Distribution of posterior probabilities across sites at node 1 for different models of ancestral reconstruction used.

within the entire dataset. This method allowed us to find a sequence of interest even when embedded within a longer AST. Database searches showed that *D. punctata* (Dippu) AST 2 (amino acids 11–18) and AST 6 occurred most frequently. However, using a degenerate search changed peptide frequency. When one amino acid site was allowed to vary from the input sequence, Dippu-ASTs 2- and 6-like sequences were the more frequent (Fig. 4B). When two amino acids were allowed to vary, Dippu-AST 6-like sequences are found in all but two insect species and all but one crustacean species (data not shown). The most frequent of all ASTs using the degenerate search method was Dippu-AST 1; this peptide appears in 372 of the 431 ASTs when two sites vary from the input. This likely reflects the length of the sequence; Dippu-AST 1 (LYDFGL) would be contained in nearly any AST as its sequence is within two amino acids of the core AST motif (Y/F)XFG(L/I).

3.3. Radiochemical assay for JH release

To ascertain if the ancestral reconstruction approach to AST analysis had physiological relevance, several peptides were synthesized and assayed for their ability to inhibit JH release by the corpora allata. AST 7 from ancestral cockroach nodes 12 and

14 were tested in *D. punctata* and *P. americana*. In both species, the cockroach ancestor, the ancestral peptide from node 14, was less potent than the extant AST 7 ($EC_{50} = 1.57 \times 10^{-8}$ M and 3.41×10^{-9} M for *D. punctata* and *P. americana*, respectively) (Fig. 5A). The Blattidae ancestor, the ancestral peptide at node 12, demonstrated potency equivalent to that of the extant AST 7 of *D. punctata* with an EC_{50} of 1.81×10^{-9} M (Fig. 5A). The Blattidae ancestor is identical to the extant AST 7 in *P. americana* and was not tested in this species as it has been tested previously (Weaver et al., 1994). For the insect dataset, peptides corresponding to AST 7 inferred for the insect and cockroach ancestors were synthesized and tested only in *D. punctata*. Here, the insect ancestor, from node 1, was a far more potent inhibitor of *in vitro* JH release than the truncated cockroach ancestor at node 3, with EC_{50} values of 1.85×10^{-9} and 2.63×10^{-8} M, respectively (Fig. 5C).

4. Discussion

Our results demonstrate that in experimental assays of inhibition of JH release, our reconstructed ancestral peptides (that are homologs of AST 7) are highly potent at the ancestral insect node, less potent at the ancestral cockroach node and show

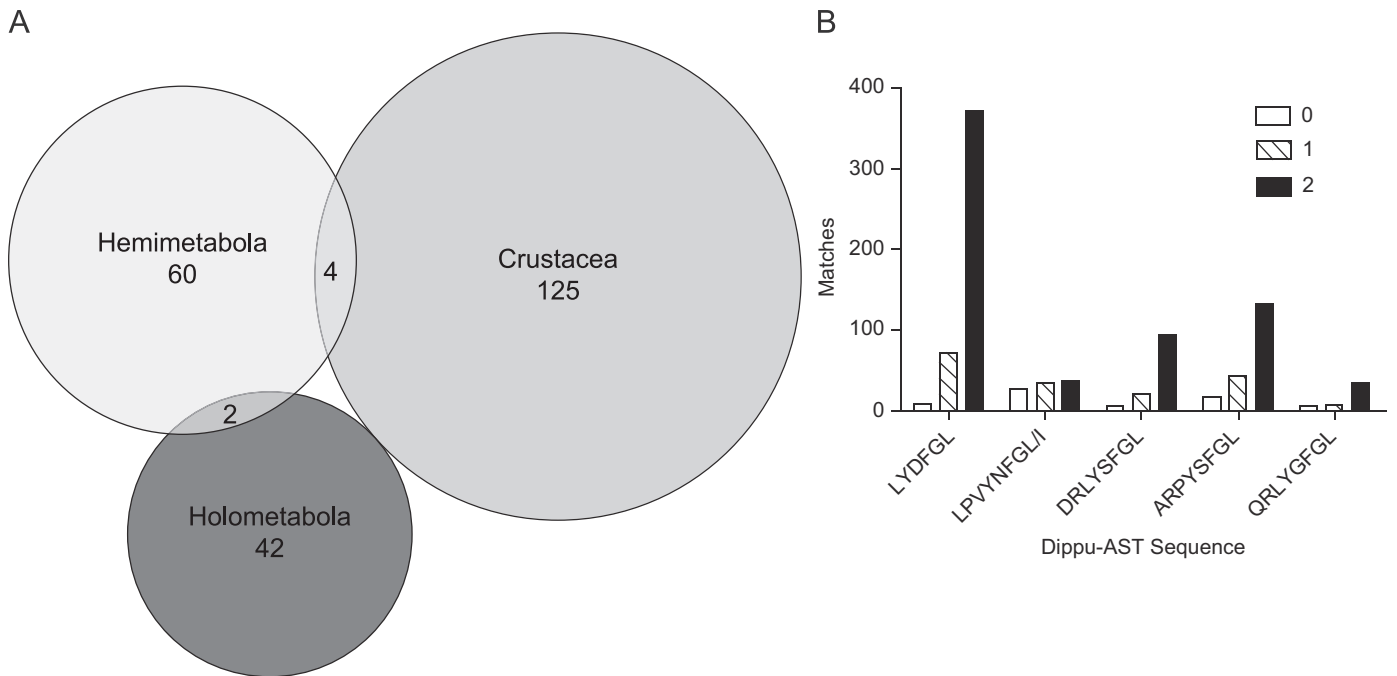


Fig. 4. Analysis of arthropod AST sequence data. (A) Proportional Venn diagram showing the number of different FGLa type AST-like peptides known in arthropod groups. The diameter of each circle is proportional to the size of the dataset for each group; overlapping regions indicate identical ASTs shared between groups. (B) AST frequency analysis using degenerate database searches of Dippu-AST sequences. The number of sequences within the dataset that match the input sequence are shown for identical sequences [0], sequences with one amino acid variation from the input [1], and sequences with two amino acid variations from the input [2].

increased potency again in more recent cockroach ancestors. To ensure that our results were not simply artefacts of the experimental system, we assayed the ancestral peptides in two different species of cockroaches (*D. punctata* and *P. americana*). The trend of high potency for the more recent cockroach and most ancient insect ancestral nodes, with decreased potency in the cockroach ancestor, was found for assays in both species. This trend, of increased potency in terms of the inhibition of JH production, appears to have occurred by two different pathways in cockroaches, with different sets of amino acid substitutions occurring in the two lineages (Fig. 1).

The question then arises as to why there is a decrease and subsequent increase in the potency of ASTs in the cockroach ancestors? Generally speaking, ASTs are thought to have increased dramatically in number, particularly within hemimetabolous insects, and diversified from a much smaller set of ancestral sequences (Bellés et al., 1999; Bendena et al., 1999; Tobe and Bendena, 1999). Although duplicated AST peptides have obvious differences from duplicated genes in that they are transcribed as one unit, once processed, the peptides may be involved in separate physiological pathways, and therefore it may be useful to consider their evolution in light of current theories of gene duplication. A classic prediction of gene duplication theory is that duplicated genes will confer redundancy, and thus allow for the accumulation of deleterious mutations (Ohno, 1970; Prince and Pickett, 2002). The early history of cockroaches appears to have been accompanied by a dramatic increase in AST peptide copy number; cockroach precursors contain 13–14 ASTs whereas holometabolous precursors only contain between four and nine ASTs and the crustacean outgroup, *C. finmarchicus*, contains seven (see Table S1). The newfound redundancy at this point in cockroach evolutionary history may explain the decreased potency of the cockroach ancestor AST 7. It is also important to note that downstream effectors of AST peptides such as the AST receptor are thought to exist in multiple forms, and thus may have

contributed to the expansion of AST copy number, but these receptors have only recently been identified, and little is known of their structure and function (Tobe and Bendena, 2006; Bendena et al., 2008; Auerswald et al., 2001; Lungchukiet et al., 2008).

Gene duplication theory also predicts that duplicates of multifunctional genes may be preserved, as different duplicates specialize for different functions over time (Wistow, 1993; Force et al., 1999), and recent surveys suggest that multifunctional genes are quite common (Piatigorsky, 2007). Experimental studies have shown that additional functions for ASTs occur in the Hemimetabola, and coincide with changes in peptide copy number. There is general agreement that myomodulatory functions of FGLa ASTs are the most widespread and likely the most ancient (for review see Bendena et al., 1999; Stay and Tobe, 2007; Tobe and Bendena, 1999). In hemimetabolous groups, which possess a large number of ASTs, ASTs serve several other functions such as the regulation of JH production, gut enzyme activity and vitellogenin production (Stay and Tobe, 2007; Martin et al., 1996; Fusé et al., 1999). This model is also supported by the situation in *S. gregaria*, a hemimetabolous insect with a lower peptide copy number of 10, in which ASTs do not regulate JH production (Vanden Broeck et al., 1996; Veelaert et al., 1996). It is also of interest that recent genomic studies of the holometabolous insect, *Tribolium castaneum*, have demonstrated that both the AST precursor and receptor genes are absent. Accordingly, gains and losses in neuropeptide genes may be fairly common (Li et al., 2008). Future work on this system should assay the capacity of ancestrally reconstructed ASTs to efficiently perform these other functions; the decreased potency of AST 7 at the cockroach ancestor node could possibly reflect its specialization on another function at that point in evolutionary history. Ideally, these studies would be performed in conjunction with reconstructions of the ancestral AST receptor in order to investigate the evolutionary history of interactions between ASTs and their receptors. Other G protein-coupled receptors have been

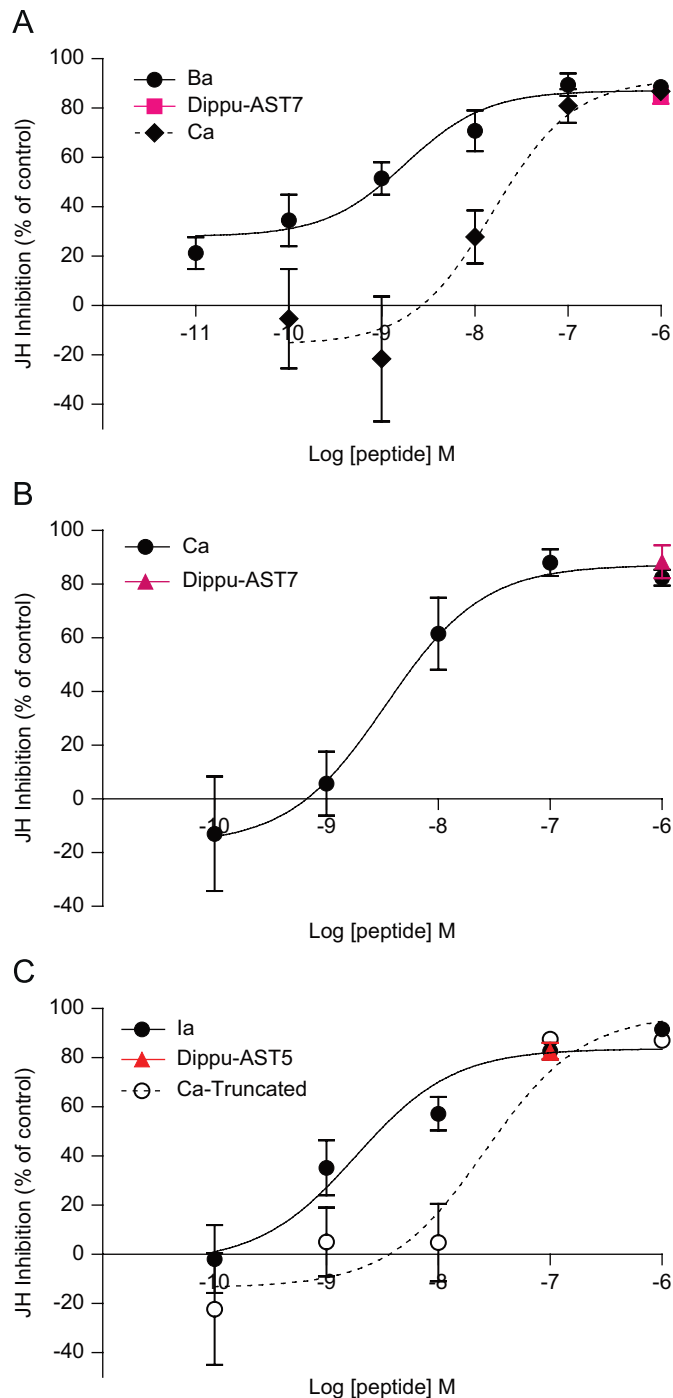


Fig. 5. Dose–response of individual corpora allata (CA) to ancestral peptides. (A) Inhibition of JH release from CA of day 7 mated female *D. punctata* following incubation with Dictyopteran ancestral peptides: Blattidae ancestor (Ba) from node 12 ($N = 10$) and cockroach ancestor (Ca) from node 14 ($N = 10$ –18). (B) Effect of Ca on the JH release from day 4 virgin female *P. americana* CA ($N = 5$ –10). (C) The effect of ancestral insect peptides on JH release from the CA of day 7 mated female *D. punctata*, Insect ancestor (Ia) from node 1 ($N = 13$ –41) and truncated cockroach ancestor (Ca-Truncated) from node 3 ($N = 16$ –41). Dippu-ASTs (5 or 7) were used as positive controls and all error bars indicate standard error.

cockroach lineages. This increased potency of ancestral cockroach ASTs may reflect the changes in reproductive biology within cockroach lineages. In these species, the timing of JH production is known to be well coordinated with reproductive events and the regulation of JH production is required for this timing (Tobe and Stay, 1977). In cockroaches, there is often a correlation between corpora allata activity and the gonadotrophic cycle whereby cycles of JH biosynthesis occur during vitellogenesis, although these cycles differ in pattern between species (Tobe and Stay, 1977). In contrast to the role of ASTs in the regulation of JH biosynthesis in cockroaches, a clear correlation of corpora allata activity with vitellogenesis is not observed in *S. gregaria* (Tobe and Pratt, 1975). In *D. punctata*, the only known viviparous cockroach species, the situation is exaggerated because here, the precise regulation of JH production is critical; the presence of JH during pregnancy results in abortion (Stay and Lin, 1981). This shift towards more complex reproductive modes in cockroaches could explain the corresponding shift in the importance of the ASTs as regulators of JH biosynthesis with respect to reproductive success and may account for the increased potency of more recent peptide ancestors.

Our database analysis of ASTs demonstrates that there is a far greater number of ASTs than previously thought. Previous estimates range from 50 to 150, whereas we show here that over 431 sequences are known (Kai et al., 2006; Bendena et al., 1999; Mousley et al., 2005; Liu et al., 2006). Interestingly, the peptides we were able to align within the insect precursor genes, according to positional homology, correspond to the peptides with the highest frequency found using our database searches. Of particular interest is the copepod *C. finmarchicus*; its AST precursor is unlike all other crustaceans previously sequenced, and is the most basal arthropod AST sequenced to date. It contains very few peptides, nearly all of which bear sequence similarity to the ASTs conserved in our insect alignments and the peptides with greatest frequency in our database. While this type of frequency-based analysis cannot determine which set of ASTs was present in the ancestral condition, it is ideal for finding peptide patterns when positional homology is lacking. This approach will be valuable as a starting point for future molecular and physiological studies.

There are several caveats which must be applied to the present work. First, there is the importance of downstream effectors in the signal cascade of ASTs. Assay of the extant *D. punctata* AST 7 in *P. americana* demonstrated that the peptide lost potency, with the EC_{50} decreasing from 1.94×10^{-9} to 6.9×10^{-8} M (Tobe et al., 2000; Weaver, 1991). Such differences in activity for a given peptide must be the result of downstream signaling events or differences in receptor specificity and represent a great unknown in AST research. Few FGLa AST receptors are known: in *Drosophila*, two receptors specific to ASTs have been identified, but they share only 47% overall sequence similarity (Birgöl et al., 1999; Lenz et al., 2001). Orthologs of the *Drosophila* receptors have been identified in *P. americana*, *B. mori*, *A. mellifera*, *A. gambiae*, *C. elegans* and recently in *D. punctata* (Auerswald et al., 2001; Gäde et al., 2008; Tobe and Bendena, 2006; Bendena et al., 2008; Lungchukiet et al., 2008). Aside from these receptors, little is known with regard to the signal transduction cascade for the AST signal, and given the multiple actions of ASTs, there are likely to be multiple pathways. While it is clear that amino acid changes in peptide sequence would affect activity, this study highlights the importance of these other signaling events. Our tests were primarily conducted in *D. punctata*, a highly derived species in which precise regulation of JH biosynthesis is particularly critical, and the ancestral AST peptides assayed here may in fact have different effects in other species.

Second, there is some contention concerning the evolutionary history of the role of ASTs in the regulation of JH biosynthesis.

successfully reconstructed and studied in this manner (Chang et al., 2002; Kuang et al., 2006).

Although function and copy number support the observed decrease in peptide potency, the question remains as to why the ASTs gained functional importance over evolutionary time in the

JH is not present in all groups in which ASTs occur; for example in Crustacea, the pathway for sesquiterpenoid biosynthesis terminates with methyl farnesoate, the immediate precursor of JH (Tobe and Bendena, 1999). Despite the altered pathway, and the absence of JH, studies have shown that AST peptides have a role in this system. Kwok et al. (2005) demonstrated that ASTs stimulate the production of methyl farnesoate by the mandibular organ, the endocrine gland homologous to insect corpora allata, in the crayfish *P. clarkii*. This is similar to the action of ASTs in early embryonic development of *D. punctata*, which has led to the suggestion that sesquiterpenoid regulation may have been an early function of ASTs in arthropods (Stay et al., 2002). However, no other crustacean species have been assayed to date (Stay and Tobe, 2007).

5. Conclusions and future directions

Ancestral reconstruction methods serve as powerful tools for investigating protein structure and function. Here, we have shown that these methods can be successfully applied to a peptide hormone system, an approach that has never been used in this context. However, to resolve broad questions about the origin of ASTs, more data will be needed both in terms of sequence and physiology. We were not able to assay any of the reconstructed peptides in terms of myoinhibition, nor were we able to test their action in higher insects. Such studies will be essential in the future. In particular, more data from primitive arthropod groups will be needed before we can attempt to determine which ASTs were part of the ancient complement of peptides. As the dataset of known ASTs grows in size, so too will the accuracy of reconstruction methods. They will no doubt prove invaluable for the future study of AST evolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinsphys.2008.04.007.

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