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# Coleoptera genome and transcriptome sequences reveal numerous differences in neuropeptide signaling between species

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**Background.** Insect neuropeptides are interesting for the potential their receptors hold as plausible targets for a novel generation of pesticides. Neuropeptide genes have been identified in a number of different species belonging to a variety of insects. Results suggest significant neuropeptide variation between different orders, but much less is known of neuropeptidome variability within an insect order. I therefore compared the neuropeptidomes of a number of Coleoptera. **Methodology.** Publicly available genome sequences, transcriptomes and the original sequence data in the form of short sequence read archives (SRAs) were analyzed for the presence or absence of genes coding neuropeptides as well as some neuropeptide receptors in seventeen beetle species.

**Results.** Significant differences exist between the Coleoptera analyzed here, while many neuropeptides that were previously characterized from *Tribolium castaneum* appear very similar in all species, some are not and others are lacking in one or more species. On the other hand, leucokinin, which was presumed to be universally absent from Coleoptera, is still present in non-Polyphaga beetles. **Conclusion.** The variability in neuropeptidome composition between insect species from from the same insect order may be as large as the one that exists between species from different orders.

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4 **Coleoptera genome and transcriptome sequences**  
5 **reveal numerous differences in neuropeptide signaling**  
6 **between species**

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17

## 18 Abstract

19

20 **Background.** Insect neuropeptides are interesting for the potential their receptors hold as  
21 plausible targets for a novel generation of pesticides. Neuropeptide genes have been identified  
22 in a number of different species belonging to a variety of insects. Results suggest significant  
23 neuropeptide variation between different orders, but much less is known of neuropeptidome  
24 variability within an insect order. I therefore compared the neuropeptidomes of a number of  
25 Coleoptera.

26 **Methodology.** Publicly available genome sequences, transcriptomes and the original  
27 sequence data in the form of short sequence read archives (SRAs) were analyzed for the  
28 presence or absence of genes coding neuropeptides as well as some neuropeptide receptors in  
29 seventeen beetle species.

30 **Results.** Significant differences exist between the Coleoptera analyzed here, while many  
31 neuropeptides that were previously characterized from *Tribolium castaneum* appear very  
32 similar in all species, some are not and others are lacking in one or more species. On the other  
33 hand, leucokinin, which was presumed to be universally absent from Coleoptera, is still  
34 present in non-Polyphaga beetles.

35 **Conclusion.** The variability in neuropeptidome composition between insect species from  
36 from the same insect order may be as large as the one that exists between species from  
37 different orders.

38

## 39 Introduction

40 Many neuropeptide signaling systems are commonly found in both protostomian and  
41 deuterostomian species, showing that most neuropeptides originated very early (*e.g.* Elphick,  
42 Mirabeau & Larhammar, 2018). Indeed it is well established that genes coding neuropeptides and  
43 their receptors are well conserved during evolution and this is not surprising as they are important  
44 regulators of a variety of physiological processes.

45 Neuropeptide evolution consists of two phenomena, the gain of novel neuropeptides and  
46 the loss of existing ones. When one compares the neuropeptidomes of decapod crustaceans with  
47 those of insects, it becomes apparent that few new neuropeptides have evolved since the  
48 existence of their last common ancestor, but that in insects a relatively large number of  
49 neuropeptide genes has been lost (Veenstra, 2016a). It would be interesting to have a better  
50 understanding of neuropeptide loss in order to get a better perspective on how it is possible that  
51 very ancient and well conserved regulatory systems can be lost in some species but remain  
52 apparently essential for others.

53 *Tribolium castaneum* was one of the first beetle species for which a complete genome  
54 sequence was published (Richards et al., 2008). As the genes coding neuropeptides and their  
55 receptors were identified it became clear that at least three neuropeptide genes that seemed to be  
56 universally present in insects, *i.e.* those coding for corazonin, leucokinin and allatostatin A, were  
57 absent from this species (Li et al., 2008). An observation that was confirmed by the absence of  
58 genes coding for the receptors of these neuropeptides (Hauser et al., 2008). The genes for two  
59 other well known insect neuropeptides, pigment dispersing factor (PDF) and neuropeptide F  
60 (NPF) were neither found in this genome (Li et al., 2008), although receptors for such peptides

61 were identified (Hauser et al., 2008). It thus appeared that the sequences of the latter two peptides  
62 might have evolved so much, that they can no longer be easily identified based on sequence  
63 homology using the BLAST program. This raises the question as to whether these peculiarities,  
64 *i.e.* the absence of three common insect neuropeptides and the apparent structural modification of  
65 two others, are characteristic of all Coleoptera and thus characteristic of this insect order, or  
66 whether they are more limited and specific for this particular species or family.

67 The public genome sequences for sixteen Coleoptera species (Richards et al., 2008;  
68 Keeling et al., 2013; Cunningham et al., 2015; Vega et al., 2015; McKenna et al., 2016; Meyer et  
69 al., 2016; Tarver et al., 2016; Ando et al., 2018; Fallon et al., 2018; Gautier et al., 2018;  
70 Kraaijeveld et al., 2018; Schoville, 2018; van Belleghem et al., 2018a,b; Wu, Li & Chen, 2018)  
71 should make it possible to identify their complete neuropeptidomes and answer this question.  
72 Given that *Tenebrio molitor* once was the most studied beetle, as still evidenced by the number of  
73 publications that can be retrieved for this species on PubMed, I have added it to the list, even  
74 though there are only transcriptome data available for this species.

75 Neuropeptides act through receptors and these may also be lost or amplified. In  
76 Chelicerates several neuropeptide G-protein coupled receptors (GPCRs) are amplified multiple  
77 times (Veenstra, 2016c). Yet I have not systematically checked whether neuropeptide receptors  
78 might be duplicated or lost. In the absence of a neuropeptide gene duplication, receptor  
79 duplication is likely to fine-tune the effects of its ligand, but this is difficult to establish. The  
80 fruitfly is no doubt the best studied insect species and while it is known to have two different  
81 allatostatin C receptors, the physiological significance of having two instead of one, like most  
82 insect species, is unknown. Therefore, receptors were only studied when the ligand appeared to  
83 be absent and in those cases where a neuropeptide gene was duplicated.

84

## 85 **Materials & Methods**

### 86 ***Definition of neuropeptide***

87 The definition of neuropeptide is sometimes ambiguous as in principle any peptide from  
88 the nervous system could be called a neuropeptide. In this manuscript neuropeptide is defined as  
89 a peptide or protein that is either released into the the hemolymph, directly on a target tissue, or  
90 within the nervous system to regulate cellular activity by interaction with a specific cell surface  
91 receptor, usually a GPCR. A large number of such neuropeptides has been identified by  
92 biological activity on target tissues and/or by directly activating their receptors, while others been  
93 identified only by their homology to known neuropeptides. Some neuropeptides have been  
94 identified solely on the basis of being produced after proteolytic processing of proteins of  
95 unknown function or even only on the basis of the strong likelihood that their putative precursors  
96 could be processed by neuroendocrine convertases into neuropeptides. The latter are hypothetical  
97 neuropeptides only and are more properly called putative neuropeptides. Indeed, a recent analysis  
98 of one such putative neuropeptide precursor in *Locusta migratoria* suggests that it is not a  
99 neuropeptide after all (Veenstra, 2017). These putative neuropeptide precursors have been  
100 included here, even though no physiological effects have been described for these peptides and  
101 their receptors are unknown. On the other hand, I have not included the putative antidiuretic

102 peptide identified from *Tenebrio* (Eigenheer et al., 2002). The definition given above does not  
103 exclude it from analysis, but it is almost certainly derived from a cuticle protein (CAA03880).  
104 Although there is a one amino acid difference between the C-terminus of the reported sequence  
105 of this cuticle protein (Mathelin et al., 1998) and the antidiuretic peptide that was sequenced,  
106 when constructing a *Tenebrio* transcript with Trinity using the various RNAseq SRAs from this  
107 species the C-terminus of this proteins was found to be completely identical to the antidiuretic  
108 peptide. There are no structure activity data with regard to its antidiuretic activity and and it is  
109 unclear which protease is responsible for cleaving it from the rest of the protein. This makes it  
110 difficult if not impossible to reliably predict which other proteins might be the precursors of  
111 similar antidiuretic peptides.

## 112 **Sequence data**

113 Genome assemblies were downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/genome>), with  
114 the exception of the genomes of *Hycleus cichorii* and *H. phaleratus*, which were obtained from  
115 the GigaScience repository (<http://gigadb.org/dataset/100405>) and those of *Photinus pyralis*,  
116 *Aquatica lateralis* and *Ignelater luminosus*, which were downloaded from <http://fireflybase.org>.  
117 When available, protein sequences predicted from the various genomes were also downloaded  
118 from NCBI or the two websites mentioned. Predicted proteins for *Hypothenemus hampei* were  
119 obtained from <https://genome.med.nyu.edu/coffee-beetle/cbb.html>, and those for *Aleochara* the  
120 Animal Ecology department of the Free University of Amsterdam  
121 (<http://parasitoids.labs.vu.nl/parasitoids/aleochara/data.php>). For *Pogonus chalceus* the published  
122 transcriptome was useful (van Belleghem et al., 2012). To facilitate reading, species will be  
123 identified by their genus name throughout this paper. In the case of the *Hycleus* species, this will  
124 refer to *H. phaleratus*. There are also two *Harmonia* genomes, but these are from the same  
125 species and they showed no differences in the genes coding neuropeptides. Four other  
126 Coleopteran genomes are publicly available, however they are not yet officially published and for  
127 this reason were not fully analyzed here. Those are *Sitophilus oryzae*, *Diabrotica viriginfera*,  
128 *Onthophagus taurus* and *Agrilus planipennis*.

129 *Pogononus* belongs to the Adephaga, all the other species to the Polyphaga suborder. The  
130 genera *Coccinella*, *Harmonia*, *Hypothenemus*, *Dendroctonus*, *Anoplophora*, *Leptinotarsa*,  
131 *Aethina*, *Hycleus*, *Tenebrio* and *Tribolium* all belong to the infraorder Cucujiformia. As will be  
132 seen this group shares certain neuropeptidome characteristics that are absent from the other  
133 Polyphaga as well as *Pogonus*.

134 The quality of these genomes is quite variable. Some have excellent assemblies and in  
135 addition numerous RNAseq SRAs making it possible to have high quality assemblies, others are  
136 much more limited. For example, the *Aleochara* assembly has no RNAseq data and only a limited  
137 amount of genomic sequences. In the case of *Aleochara* there is RNAseq data from a different  
138 species, *A. curtula* (SRR921563, from the 1KITE project, Misof et al., 2014), which was helpful  
139 and it allowed in some case to reconstruct exons missing from the assembly using a combination  
140 of raw genome sequences and trinity. Nevertheless, it is still possible to ascertain the presence or  
141 absence of neuropeptide genes from this assembly.

142 In several instances the predicted complete coding sequences of some neuropeptides are  
143 incomplete. When there is little RNAseq data to deduce precursor sequences and a draft genome  
144 contains large and small gaps in the assembly such sequences are often incomplete and may well  
145 be incorrect in the parts that have been deduced. The *Oryctes* and *Aleochara* draft genomes suffer  
146 the most from these problems.

147 A complete list of all SRAs used is available as supplementary data (Table S1).

### 148 **Presence of neuropeptide and receptor genes**

149 Predicted neuropeptide precursors were preferentially obtained from the annotated  
150 genomes, but this was not always possible. On the one hand, small neuropeptide genes are often  
151 overlooked by automated annotation programs, even though progress has been quite impressive  
152 in that respect, on the other hand there are quite a few transcripts that are probably wrong. Thus  
153 many neuropeptide precursors were corrected or predicted *de novo* from RNAseq data by using  
154 the `tblastn_vdb` command from the SRA Toolkit  
155 (<https://www.ncbi.nlm.nih.gov/sra/docs/toolkitsoft/>) on one or more SRAs using the *Tribolium*  
156 neuropeptide precursors as query to extract reads that could potentially encode a homologous  
157 protein. Those reads were then assembled using Trinity (Grabherr et al., 2011) and transcripts  
158 that might encode neuropeptides or other proteins of interest were then identified using BLAST+  
159 (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast/>). Trinity produced transcripts were judged  
160 complete when the N-terminal of the predicted neuropeptide precursors had a signal peptide that  
161 could be identified as such by SignalP (Petersen et al. 2011) as implemented on the web  
162 (<http://www.cbs.dtu.dk/services/SignalP/>) and had an inframe stop codon at the C-terminus. For  
163 G-protein coupled receptors (GPCRs) the identification of the N-terminus is often more  
164 ambiguous as some sequences did not have an in-frame stop codon upstream from the putative  
165 ATG start codon. In those cases perceived similarities with homologous GPCRs were used as  
166 criterium for completeness. However, for the GPCRs analyzed here the aim was not to obtain  
167 absolutely perfect sequences for each receptor, but rather to show whether or not it is present in a  
168 particular species.

169 When in a first round of analysis with the `tblastn_vdb` command incomplete sequences  
170 were obtained, partial transcripts were then used as query for the `blastn_vdb` command to obtain,  
171 where possible, the remainder of the putative transcripts. This process sometimes needed to be  
172 repeated multiple times. Transcripts could also be completed by using the assembled genomes,  
173 and in several instances no transcripts were obtained at all and only the genome was available.  
174 Although many genes were located on single genomic contigs, this was not always the case. In  
175 those cases either Trinity produced transcripts and/or individual RNAseq reads, or homology  
176 with other precursors from other species were used to confirm the continuity of these transcripts.

177 It may be noted here that not all trinity produced transcripts are copies of mRNA species  
178 of the genes their sequences seem to indicate. In a previous paper on the RYamide gene in  
179 *Drosophila melanogaster* we showed that the very large majority of RNAseq reads that  
180 correspond to the coding sequence of this neuropeptide in the various SRAs are not due to the  
181 transcription of the RYamide gene, but rather parts of 3'-ends of mRNA produced from genes  
182 located upstream (Veenstra and Khammassi, 2017). The RYamide gene is very little expressed



183 in *D. melanogaster* and mostly in only two neurons in the adult. This causes the RYamide  
184 transcript to be so rare that virtually every RNAseq read that contains part of the coding sequence  
185 of this gene is in fact an artifact and is not derived from an authentic RYamide mRNA. It is likely  
186 that RNAseq reads from other genes that are neither extensively expressed, such as is the case for  
187 many neuropeptide genes, may similarly be the product from a heavily expressed upstream gene,  
188 rather than from the neuropeptide gene in question. This problem was *e.g.* encountered with the  
189 PTTH gene from *Hycleus* and the *Leptinotarsa* periviscerokin gene. Such RNAseq reads attest  
190 to the existence of the neuropeptide gene in question, but have not necessarily undergone the  
191 same splicing as the one that is imposed on the neuropeptide mRNA. This phenomenon explains  
192 why certain Trinity produced transcripts predict mRNA sequences that contain introns that have  
193 not been excised. Whereas in some cases such “false” transcripts can be discarded easily due to  
194 the presence of an in-frame stop codon, in other instances such stop codons are absent. Even  
195 though obviously such data reveal alternatively splicing, it is not at all clear that this alternatively  
196 splicing actually occurs in mRNAs produced from the neuropeptide gene. In other words, what at  
197 a first impression may look like very sloppy intron processing, may in fact reflect sloppy stop  
198 codon processing in a gene upstream where such sloppiness has no consequences. It is for this  
199 reason that I have made no effort to carefully analyze all alternative splice forms for neuropeptide  
200 precursors and only recorded those that seem authentic and physiologically relevant.

201 The presence in RNAseq data of sequences that represent 3'-ends of primary mRNA  
202 sequences in which the polyadenylation signal has been ignored may lead to Trinity transcripts  
203 that are longer and extend into downstream genes. Thus many Trinity produced transcripts appear  
204 at first sight to lack a signal peptide, such transcripts were corrected by removing sequences  
205 judged to be extraneous based on sequence homology with other species and in the case of  
206 neuropeptide precursors on the presence of a credible signal peptide.

### 207 **Absence of neuropeptide and receptor genes**

208 The methodology described above allows one to demonstrate the presence of particular  
209 neuropeptide. However, when a particular gene is not identified in this way, it does not  
210 necessarily mean its absence from the species in question. When a gene is absent from the  
211 transcriptome, it may be simply because its expression levels are very low, as *e.g.* in the case of  
212 the previously mentioned RYamide gene from *D. melanogaster*. If the gene is also absent from  
213 the genome assembly, it is possible that it is located in a part of the genome that did not make it  
214 into the genome assembly.

215 Neuropeptides act via receptors, most of which are GPCRs. In many cases, but certainly  
216 not all, GPCRs are specific for a particular neuropeptide. So if a neuropeptide gene is genuinely  
217 missing from a species, one should expect its receptor to have lost its function and no longer be  
218 subject to positive selection. Hence, its receptor is expected to be lost as well. So, when both a  
219 neuropeptide and its unique receptor(s) are absent from a genome assembly, it is a good  
220 indication that the particular neuropeptide signaling system has been lost from the species in  
221 question.

222 For receptors that may be activated by neuropeptides derived from different genes, this  
223 argument can not be used. For example, a *Bombyx* myosuppressin receptor can be activated by



224 both myosuppressin and FMRFamides (Yamanaka et al., 2005, 2006), hence if either the  
225 myosuppressin or FMRFamide were lost, this receptor could still be present. Similar situations  
226 likely exists for other neuropeptides, *e.g.* the CCHamides 1 and 2 (Hansen et al., 2011; Ida et al.  
227 2012) or sNPF and NPF (*e.g.* Reale, Chatwin & Evans, 2004). Thus missing neuropeptide  
228 receptors can only be used to validate the absence of a neuropeptide ligand, if these receptors are  
229 activated exclusively by that ligand.

230 The loss of a gene can in principle only be demonstrated by flawless genome assemblies  
231 (they don't exist), however there is an alternative, that is almost perfect. It exists in the analysis  
232 of the original genomic reads obtained for the assembly. When those reads are very numerous  
233 short reads, the chance that there is not a single read that covers the gene in question becomes  
234 extremely small and thus negligible. The only remaining problem than is the question, whether or  
235 not the gene in question can be reliably identified from a single short read. For the most GPCRs  
236 the answer to this question is yes, as the sequences of the seven transmembrane regions are  
237 strongly conserved and there are always a couple of them that one can unambiguously identify as  
238 being part of a particular receptor. Obviously, this might not work if all the individual  
239 transmembrane regions of a GPCR were coded by two exons interrupted by an intron. But this is  
240 not the case for the GPCRs analyzed here. An illustration of this method is provided as a  
241 supplementary figure (Fig. S1).

242 For the analysis of the absence of neuroendocrine convertase PC1/3 a similar procedure  
243 was applied. This was relatively easy, as this protein has a very well conserved primary sequence.

244 To demonstrate the absence of a particular neuropeptide gene in this fashion is much  
245 more difficult. First, many neuropeptide genes code for a single neuropeptide and the remainder  
246 of the precursor is often too poorly conserved to be recognized reliably in short genomic reads  
247 from species that are not closely related. Secondly, in some cases the sequence coding the peptide  
248 or its most conserved parts, may be interrupted by an intron. For example, the genomic sequences  
249 coding the neuropeptide F family all have a phase 2 intron in the triplet coding the Arg residue of  
250 the C-terminal Arg-Phe-amide, making identification of genomic sequences coding this peptide  
251 more difficult. A similar intron is present in the elevenin gene. Thirdly, some neuropeptides are  
252 not only small but are also made up of amino acids that have very degenerate codons, this is the  
253 case for short NPF (sNPF). Finally, sometimes conserved amino acids in a particular  
254 neuropeptide are no longer conserved, as is the case for allatotropin in honeybees and other  
255 Hymenoptera (Veenstra, Rodriguez & Weaver, 2012). On the other hand, when dealing with a  
256 larger peptide that is structurally well conserved during evolution this would provide an  
257 additional argument for its absence.

## 258 **Sequence comparisons**

259 For comparing the sequences of various neuropeptides I have used Seaview (Gouy,  
260 Guindon & Gascuel, 2010) and the figures it produces. The different colors that are used to  
261 identify amino acid residues with similar chemical characteristics (acidic, basic, aromatic,  
262 aliphatic etc) provide good visualization of conserved amino acid sequences when absolute  
263 conservation of residues is limited.

264

## 265 Results

### 266 **General comments**

267 Neuropeptides have previously been identified and sequenced for two of the species  
268 analyzed here, the Colorado potato beetle and the meal worm. The sequences of the two sNPFs  
269 and the two AKHs that were identified from potato beetle (Gäde & Kellner 1989; Spittaels et al.,  
270 1996a) are exactly the same as those predicted from the genome. On the other hand none of  
271 *Leptinotarsa* neuropeptide genes predict the same structure as the allatotropin ortholog from  
272 *Locusta migratoria* and while this species has a proctolin gene, it does not predict an [Ala<sup>1</sup>]-  
273 proctolin. Two other peptides that were reportedly isolated from this species could neither be  
274 identified in any of the genomic or transcriptomic sequences analyzed here (Spittaels et al., 1991;  
275 1995a,b; 1996b).

276 The sequences of *Tenebrio* AKH, myosuppressin, three pyrokinins, DH37 and DH47 as  
277 predicted here from the transcriptome are identical to those reported previously, the only  
278 difference being that the transcriptome suggests a C-terminal amide for DH47 instead of the  
279 reported C-terminal acid (Gäde & Rosiński, 1990; Furuya et al., 1995, 1998; Weaver & Audsley,  
280 2008; Marciniak et al., 2013).

281 The majority of the neuropeptide precursors seem quite similar in structure between the  
282 different species. Those will not be commented upon, but their sequences can be found in the  
283 supplementary data (Table S2; Supplementary Figures). To facilitate interpretation of the data  
284 several figures include a simplified phylogenetic tree of the species analyzed. This tree is based  
285 on the extensive phylogenetic tree recently published for Coleoptera (Zhang et al., 2018). When I  
286 use the term closely related species in the text, this is short hand for species that are neighbors on  
287 the simplified phylogenetic trees.

### 288 **Significant changes in peptide sequences**

289 PDF, pigment dispersing factor

290 The PDF present in *Tribolium* and other Cucujiformia has two more amino acid residues  
291 than the *Drosophila* peptide and differs from it especially in its C-terminal half (Fig. 1). This  
292 explains why it wasn't identified in a previous study (Li et al., 2008). In the other Polyphaga it is  
293 more similar to the *Drosophila* peptide, but in *Polygonus* it is two amino acids shorter than in  
294 *Drosophila*.

295 NPF, neuropeptide F

296 The structure of NPF has changed even more than that of PDF. It is relatively common for  
297 a Phe to residue change into a Tyr and *vice versa* and so the mutation of the C-terminal Arg-Phe-  
298 amide into Arg-Tyr-amide in most species studied here, is not unusual. More drastic is the  
299 presence of disulfide bridge in the N-terminal of NPF in the Cucujiformia and the mutation of the  
300 C-terminal Arg-Tyr-amide into a Pro-Tyr-amide in the two Curculionids. The primary sequence  
301 similarity of the predicted peptides to each other and other insect NPFs, as well as the

302 characteristic phase 2 intron in the Arg residue (Pro in the Curculionids) of the C-terminal of  
303 these peptides confirm that these are true NPF orthologs (Fig. 2). I was unable to find an *Oryctes*  
304 NPF gene, although an NPF receptor seems to be still present in this species. Given the enormous  
305 structural variability of this peptide in Coleoptera it is not clear whether this is because the NPF  
306 gene was lost, or whether in this species the peptide has undergone even larger sequence changes.

### 307 ACP

308 ACP is a peptide that has been lost independently at least three times and in those species  
309 where the gene is still present the predicted peptide sequences are quite variable (Fig. 3).

### 310 Baratin or NVP-like precursor

311 Baratin is a small neuropeptide initially isolated from the cockroach *Leucophaea maderae*  
312 (Nässel, Persson & Muren, 2000) that has been shown to be produced from a large neuropeptide  
313 precursor that has been called NVP-like in *Tribolium* (Li et al., 2008). This neuropeptide  
314 precursor is well conserved in Coleoptera (Fig. S2), except that in *Dendroctonus* it is lacking the  
315 last part as deduced from both in the genome and transcriptome sequences [note there is another  
316 baratin precursor at NCBI that is supposedly also from *Dendroctonus*, however analysis of the  
317 various SRAs from which this transcriptome is made shows that one of them (SRR2044898)  
318 contains in addition to *Dendroctonus* a second unidentified species from which this baratin  
319 precursor transcript originates].

### 320 Calcitonin B

321 The *Leptinotarsa* and one of the *Anoplophora* calcitonin genes encode not only typical  
322 calcitonin peptides, but also a number of structurally very similar peptides that lack the disulfide  
323 bridge in the N-terminal portion of the molecule (Fig. 4).

### 324 Elevenin

325 Like ACP, elevenin has been lost independently at least three times and in those species  
326 where this gene is still present the predicted elevenin sequences are also very variable (Fig. 5).

### 327 Myosuppressin

328 Myosuppressin is always located at the very end of its precursor and in virtually all insect  
329 species after the Gly residue that will be transformed in the C-terminal amide the precursors ends  
330 with two, three or occasionally four dibasic amino acid residues. Surprisingly this is not so in  
331 Coleoptera, where all myosuppressin precursors terminate with a few additional amino acid  
332 residues after those dibasic amino acid residues (Fig. S3).

### 333 Orcokinin convertase cleavage sites

334 In those species where the organization of the exons of the orcokinin gene could be  
335 established, it was similar to the one described previously for other insects (*e.g.* Veenstra & Ida,  
336 2014). Due to the presence of numerous copies of orcokinin B peptides, sequences of this gene  
337 are usually very difficult to assemble using short reads and this explains the problems with the

338 orcokinin genes of *Oryctes* and *Aleochara*, although in both cases the presence of orcokinin was  
339 established. What makes these genes interesting is the convertase cleavage sites in the orcokinn B  
340 precursors. Proteolytic processing of neuropeptide from their precursors occurs at specific dibasic  
341 amino acid residues, usually a Lys-Arg pair. When processing occurs at single Arg residues, as is  
342 the case for most orcokinin B precursors, empirical rules describe that other dibasic amino acid  
343 residues need to be located nearby in the precursor (Veenstra, 2000). However, orcokinin B  
344 precursors do not conform, which suggests that they are processed by a different convertase than  
345 the one processing the majority of insect neuropeptide precursors. Interestingly, in the two  
346 Coccinellids studied here, *Harmonia* and *Coccinella*, as well at least another, *Serangium*  
347 *japonicum* (GGMU01110504.1), the convertase cleavage sites have been replaced by the more  
348 classical Lys-Arg sites. In *Aethina* a few single Arg cleavage sites are still present, but the  
349 majority are also Lys-Arg pairs (Fig. S4). This suggested that this second convertase may have  
350 been lost. The two most common neuroendocrine convertases are PC1/3 and PC2; both are  
351 commonly present in insects (e.g. Veenstra, 2017), but PC1/3 is absent from *Drosophila*, a  
352 species in which the orcokinin B precursor also has Lys-Arg convertase cleavage sites (Veenstra  
353 and Ida, 2014). When looking for these two convertases in Coleoptera, it became clear that PC1/3  
354 is similarly lacking in Coccinellids but present in the other species, including the *Aethina*.

#### 355 Periviscerokinin (Capa peptides)

356 The periviscerokinins have often the typically the C-terminal sequence FPR(V/L/I)amide,  
357 but although some of the Coleoptera peptides have this sequence (Fig. S5), others have not.  
358 Detailed analysis of three receptors activated by pyrokinins, tryptopyrokinins and  
359 periviscerokinins in *Tribolium* shows that a periviscerokinin with a C-terminal LTPSLRVamide  
360 is as good a ligand as the MVSFPRIamide (Jiang et al., 2014). This analysis also reveals that  
361 none of these receptors preferentially recognizes the tryptopyrokinins, which in *Drosophila* and  
362 mosquitoes have a dedicated receptor (Cazzamali et al., 2005; Olsen et al., 2007). Unfortunately,  
363 I was unable to reconstruct a complete periviscerokinin transcript for neither *Leptinotarsa* nor  
364 *Harmonia* from either the genomic or the transcriptomic data.

#### 365 Proctolin

366 The predicted proctolin sequences of *Harmonia*, *Coccinella* and *Oryctes* deviate from the  
367 classical peptide. This is described in more detail in the following section on gene losses.

### 368 **Gene Losses**

#### 369 Unambiguous gene losses

370 There are a number of instances in which genes could not be found in the assembled  
371 genome of a species. In six cases this concerns neuropeptides with a known and unique receptor  
372 which is also absent from the same genomes that lack the genes for the ligands. It was previously  
373 reported that the *Tribolium* lacks both ligand and receptor genes for allatostatin A, corazonin and  
374 leucokinin (Li et al., 2008; Hauser et al., 2008). The first two were found to be absent from all  
375 Coleoptera studied here, while both leucokinin and its receptor were found in *Pogonus*, the only

376 species outside the Polyphaga suborder for which a genome is available. However, neither  
377 leucokinin nor its receptor was found in any of the other species. Leucokinin is also present in  
378 other species that do not belong to the Polyphaga suborder. As noted above both ACP and  
379 elevenin were lost independently at least three times, while natalisin was lost at least twice in  
380 Coleoptera (Fig. 6). Interestingly in *Photinus* there is still a remainder of the original calcitonin  
381 gene. It is clearly defective as it misses essential parts and it is no longer expressed, while the  
382 putative receptor (cf Veenstra, 2014) is completely gone. A similar situation occurs with the  
383 relaxin gene in *Sitophilus oryzae*; there also a remainder of non-functional relaxin gene is still  
384 present, but its putative receptor (cf Veenstra, 2014) is absent.

#### 385 Dilp8 orthologs

386 The structure of dilp8, *Drosophila* insulin-like peptide 8, is very poorly conserved and it  
387 has so far not been detected outside flies. LGR3 (Leucine Rich Repeat GPCR-3) has been  
388 identified as the receptor for this peptide (Vallejo et al., 2015) and this receptor, although absent  
389 from *Tribolium*, was found in a number of species (Fig. S7), suggesting that it got independently  
390 lost on at least four occasions (Fig. 6).

#### 391 Eclosion Hormone

392 Most Coleoptera have two eclosion hormone genes (Fig. S7), but the second gene appears  
393 to be missing in *Coccinella*, *Harmonia* and *Dendroctonus*, while in *Hycleus* there is still a  
394 sequence that can be recognized as once have being part of such a gene, but it is no longer  
395 functional. In many genomes the two are located on the same contig. All four possible  
396 configurations [head to head, tail to tail, one upstream from two, two upstream from one] are  
397 present, but there is no clear pattern.

#### 398 Elevenin

399 The presence of elevenin in *Oryctes* and *Aleochara* is not clear. On the one hand there are  
400 genomic sequences in *Oryctes* that code for what looks like parts of an elevenin precursor, even  
401 though the predicted elevenin peptide deviates even more from the elevenin consensus sequence  
402 than the average Coleopteran elevenin. On the other hand no traces were found of a putative  
403 elevenin GPCR. Therefore elevenin may well be also absent from *Oryctes*. A similar but different  
404 problem occurs with *Aleochara*, here a putative elevenin GPCR is present in the genome, but the  
405 elevenin precursor could not be found. This is not so surprising as its precursor is hardly  
406 conserved outside the sequence of the neuropeptide itself and even that sequence is so poorly  
407 conserved within the Coleoptera (Fig. 7) that the characteristic intron splice site inside the  
408 neuropeptide sequence is often needed to confirm that it is indeed elevenin. However, the same  
409 intron splice site makes finding homologous sequences much more difficult.

#### 410 sNPF

411 In all species an sNPF GPCR can be identified, but the sNPF precursor (Fig. S9) was  
412 found in neither the *Photinus* nor the *Aquatica* genome. These two Coccinellid species are  
413 relatively closely related and the absence of the sNPF precursor from both suggests that it was



414 already lost in their last common ancestor. It seems unlikely to be a case of genome assembly  
415 problems, as despite several efforts not a single transcriptome read could be found that could  
416 represent an sNPF mRNA. There are two possible explanations. The first one is that the sNPF  
417 precursor has been lost in these two species but its receptor is still being used by a different  
418 peptide, *e.g.* another N-terminally extended RFamide. The second possibility is that the sequence  
419 of the peptide has undergone so many structural changes, that it is now impossible to find it using  
420 the BLAST program for homology searches.

#### 421 Proctolin

422 Proctolin was the first neuropeptide for which a complete chemical structure was  
423 determined (Starratt & Brown, 1975). It is commonly present in insects, although it seems to be  
424 absent from at least some Lepidoptera and Hymenoptera (*e.g.* Roller et al., 2008; Kanost et al.,  
425 2016; Hummon et al., 2006; Hauser et al., 2006, 2010; Schmitt et al., 2015). Its pentapeptide  
426 sequence (Arg-Tyr-Leu-Pro-Thr) has been well conserved during evolution and is exactly the  
427 same in Chelicerates, Myriapods, Decapods and insects (Veenstra, 2016a,c). It is therefore  
428 interesting to see that in Coccinellids the predicted sequence of this peptide has mutated to [Ser<sup>4</sup>]-  
429 proctolin. In *Oryctes* the proctolin precursor also predicts a non-classical proctolin, in this case  
430 [Ala<sup>5</sup>]-proctolin. In all three species these sequences are deduced from both the genome and  
431 transcriptome sequences. On the other hand, the overall structures of these putative proctolin  
432 precursors are well conserved (Fig. S10). Nevertheless, no proctolin receptors could be found in  
433 either *Oryctes* or two Coccinellid species.

#### 434 Other peptides that are absent from Coleoptera

435 Calcitonin A and CCRFamide have never been found in Holometabola, and they were  
436 neither found here. In Coleoptera tryptopyrokinin coding sequences were only found as part of  
437 the periviscerokinin and pyrokinin genes and hence a specific tryptopyrokinin gene as exists in  
438 termites and locusts (Veenstra, 2014) was not found in Coleoptera. Of the three allatostatins Cs  
439 (Veenstra, 2016b) only CC and CCC were found and neither did we find any evidence for a  
440 second NPF gene. EFLamide is difficult to find, because its conserved sequence is so short  
441 (Veenstra, 2019). Insect species that have an EFLamide gene also have an ortholog of the  
442 *Platynereis* EFLamide GPCR (Bauknecht & Jékely, 2015), but such an ortholog is missing from  
443 all the Coleoptera genomes studied here.

444 The recently described putative neuropeptide precursor RFLamide (Liessem et al., 2018)  
445 is easily detectable in most Coleoptera (Fig. S12), but was not found in either of the two  
446 Curculionids, *Hypothenemus* and *Dendroctonus*. Hence, it is likely that this gene is missing from  
447 those two species as well as from other Curculionidae.

## 448 **Gene Duplications**

### 449 AKH

450 When the putative Coleopteran AKH precursors are aligned it is evident that they consist  
451 of four different regions, the signal peptide, the AKH peptide sequence with its processing site  
452 consisting of the GKR triplet, a hydrophilic connecting peptide (C-peptide) and a more  
453 hydrophobic disulfide bridge containing sequence (Cys-peptide). It is noticeable that the  
454 sequences of the signal peptides, AKHs and the Cys-peptides are very well conserved (Fig. 7),  
455 albeit that there are a number of exceptions. The most glaring examples are the second putative  
456 *Harmonia* AKH gene, which obviously can not encode an AKH, and the putative *Aethina* AKH  
457 precursor that is predicted to have no functional signal peptide.

### 458 Bursicon

459 The bursicon sequences are all very similar, as expected from this well conserved and  
460 essential insect hormone (Figs. S13 and S14). *Oryctes* is the only species that is noteworthy in  
461 that it has two bursicon A genes the start ATGs of which are 4184 nucleotides apart on the same  
462 contig. When one compares the predicted mature protein sequences, it is clear that the second one  
463 has several amino acid changes which in all the other proteins are well conserved (Fig. S13). It is  
464 impossible to know which of these two genes are most expressed, as all the Bursicon A intron  
465 splice sites in both genes are ignored by the various RNAseq reads. The only Trinity transcript  
466 generated from this genomic region that has an intron reveals that it was generated from the  
467 opposite DNA strand. So possibly all the RNAseq reads present in the only public transcriptome  
468 SRA (SRR2970555) that cover the Bursicon A genomic region of this species are generated from  
469 the opposite strand and thus originate from a different gene.

### 470 Calcitonin

471 Two genes coding calcitonins are present in *Anoplophora*, *Hycleus*, *Tribolium* and  
472 *Tenebrio*, they are described in greater detail in the section on paracopy duplication.

### 473 CCHamide 2

474 In *Leptinotarsa* the CCHamide 2 gene is duplicated (Fig. S14) and so is the CCHamide 1  
475 receptor. Phylogenetic tree analysis of CCHamide receptors shows that the two *Leptinotarsa*  
476 CCHamide 1 receptors more similar to one another than to the *Anoplophora* ortholog, thus  
477 suggesting that the duplication of this receptor is relatively recent (Fig. 8).

### 478 Insulin-related peptides

479 Insects have three different types of insulin, two of which act, predominantly or  
480 exclusively, through GPCRs, these are relaxin and the dilp8 orthologs. The third type acts  
481 through a classical tyrosine kinase receptor and in most insect species the latter insulin genes are  
482 amplified. In Coleoptera their numbers range from two in *Aquatica* and *Pogonus* to ten in  
483 *Anoplophora*. The primary amino acid sequence is in general not very well conserved, making it



484 difficult if not impossible to make reliable trees of insect insulin genes. However, it is clear that  
485 genes were lost and added on multiple occasions. The strong sequence divergence of these  
486 proteins implies that one can only make phylogenetic trees for relatively closely related species.  
487 Such a tree for *Hycleus*, *Tribolium* and *Tenebrio* (Fig. S15) shows that *Tribolium* must have lost  
488 the ortholog of the *Hycleus* insulin 3 and *Tenebrio* insulin 1 genes. A similar tree made for the  
489 insulin sequences from *Dendroctonus*, *Hypothenemus*, *Anoplophora*, *Leptinotarsa* and *Aethina*  
490 similarly shows shared ancestors for several of their insulin genes as well as recently amplified  
491 insulin genes in *Leptinotarsa*, *Anoplophora* and *Aethina* (Fig. S16).

#### 492 Myosuppressin

493 In *Leptinotarsa* the myosuppressin gene has been amplified and its genome now has four  
494 such genes, one that is producing a classical myosuppressin and three others that at first sight  
495 seem to code for a smaller analog of myosuppressin, but on the basis of the described specificity  
496 of neuropeptide convertase (Veenstra, 2000), it is also possible that they produce N-terminally  
497 extended myosuppressins, as Lys-Arg cleavage sites followed by an aliphatic amino residue are  
498 rarely cleaved, and this even more unlikely for the precursor in which the putative Lys-Arg  
499 cleavage site has mutated into a Lys-Lys site (Fig. S3). All four genes are expressed as shown by  
500 the various RNAseq SRAs. Interestingly, this gene is also amplified in the Scarabaeid  
501 *Onthophagus taurus*, where there are at least three genes that express a myosuppressin precursor.  
502 Thus the myosuppressin gene was amplified independently at least twice in Coleoptera.

#### 503 Neuroparsin

504 The neuroparsin gene is present as a single copy in all species, except *Oryctes* where it is  
505 duplicated (Fig. S17) and perhaps even triplicated, as the second gene is present in two copies in  
506 the genome assembly; those may represent either two quite divergent alleles of the same gene or  
507 perhaps more likely a gene duplication. In the RNAseq SRA of this species (SRR2970555) these  
508 three sequences are represented by 44, 19 and 11 half spots respectively).

#### 509 Pyrokinin

510 The basic beetle pyrokinin gene has three coding exons, the two introns in between are  
511 phase 1 and phase 0, which makes amplification of the intermediate coding exon, such as  
512 occurred in the periviscerokinin gene, very difficult. The first coding exon contains the signal  
513 peptide, the second a copy of tryptopyrokinin and the last one three pyrokinin paracopies. The  
514 *Pogonus* gene has three coding exons, but the precursor only codes for two pyrokinin paracopies.  
515 The tryptopyrokinin has also been lost from the Coccinellid precursors, one of two pyrokinin  
516 precursors in *Nicrophorus*, *Dendroctonus*, *Hypothenemus*, *Anoplophora* and *Leptinotarsa*, all  
517 *Photinus*, *Aquatica* and *Ignelater* precursors and probably three out of five in *Aethina*. It thus  
518 looks like that in several Coleoptera species evolution favored the production of pyrokinins over  
519 that of tryptopyrokinin from these genes. The pyrokinin gene is amplified in five of the species  
520 studied here; most of these are segmental amplifications, but in *Oryctes* there is one gene that has  
521 no longer any introns and may have originated from retroposition (Fig. S18).

## 522 Relaxin

523 The gene coding for relaxin (the ortholog of *Drosophila* insulin-like peptide 7) is  
524 duplicated in *Aethina* (Fig. S19). Both copies look like they can produce functional proteins and  
525 both genes are expressed (there are 170 and 89 reads for for the coding sequences of relaxin-1  
526 and -2 respectively in SRR1798556).

## 527 Vasopressin

528 Genes encoding vasopressin-related peptides were found in all species analyzed (Fig.  
529 S20). In all of them, except *Leptinotarsa*, these genes code for CLITNCPRG-amide, the peptide  
530 that was identified from *Locusta migratoria* (Proux et al., 1987). In the Colorado potato beetle  
531 two such genes were found and they code for two different vasopressin-like molecules  
532 CLITNCPKG-amide and its analog CLITNCPIG-amide. Interestingly, various vasopressin  
533 antisera that were used to label vasopressin-immunoreactive neurons in this species labeled the  
534 two vasopressin-specific neurons only weakly while the adjacent pyrokinin containing  
535 neuroendocrine cells that have a C-terminal PRLamide sequence intensely (see *e.g.* fig. 3 from  
536 Veenstra, Romberg-Privee & Schooneveld, 1984), while the same antisera stain the vasopressin  
537 specific cells in the *Locusta* just as intensely as the ones producing pyrokinin (Veenstra, 1984).  
538 Each of the two *Leptinotarsa* peptides could explain these results, the Lys-analog, as the Lys  
539 residue is likely to be cross-linked by formaldehyde and hence no longer immunoreactive, and  
540 the Ile-analog because it lacks the basic amino acid residue that is likely important for  
541 immunoreactivity. Counts of RNAseq reads in *Leptinotarsa* SRAs reveals twice as many reads  
542 for the Ile-analog as for the Lys-analog (652 versus 341).

543 Although there are two vasopressin genes, there is only a single vasopressin receptor  
544 present in the genome.

545 **Exon duplications**

## 546 Allatostatin CCC

547 In both *Aleochara* and *Nicrophorus*, but not in closely related *Oryctes*, the second and last  
548 coding exon of the allatostatin CCC gene has been duplicated allowing the production of two  
549 different allatostatin CCC transcripts (Fig. 9) predicted to produce slightly different allatostatin  
550 CCC peptides that both conform to the consensus sequence of this peptide (Veenstra, 2016b). In  
551 the other species only a single allatostatin CCC precursor was found (Fig. S21).

## 552 Allatotropin

553 The *Pogonus* allatotropin precursor is almost indistinguishable from the Hemimetabola  
554 sequences; it shares with them the N-terminal Gly-Phe-Lys and the remainder of its precursor is  
555 also very similar. However, in the other Coleoptera allatotropin sequences these characteristics  
556 have not been conserved (Fig. S22). On two occasions the allatotropin precursor has acquired a  
557 second allatotropin paracopy, once by adding a second exon and a second time by adding an  
558 additional allatotropin paracopy directly next to the existing one (Fig. 10).

## 559 Calcitonin

560 The calcitonin precursor is one of the most variable neuropeptide precursors in Coleoptera  
561 (Fig. S23). A functional calcitonin gene is absent from *Photinus*, where a remainder of the gene  
562 for the peptide can still be found, but where the putative receptor has completely disappeared,  
563 while in four of the other species, *Anoplophora*, *Hycleus*, *Tribolium* and *Tenebrio*, there are two  
564 calcitonin genes. The *Leptinotarsa* and one of the *Anoplophora* genes encode not only typical  
565 calcitonin peptides, but also a number of structurally very similar peptides that lack the disulfide  
566 bridge in the N-terminal portion of the molecule (Fig. 5). The number of paracopies predicted  
567 from each precursor varies from one to one to seven. The sequences of several of these precursors  
568 suggests that they have lost one or more calcitonin paracopies during evolution (Fig. S23).

## 569 DH31

570 The DH31 gene shows considerable variation in its structure and the peptides it produces.  
571 Although in all species, it codes for the classical DH31 that is very well conserved (Fig. S24), in  
572 several species additional neuropeptides are encoded on alternatively spliced mRNAs that do not  
573 encode DH31. In its most basic form the gene produces a single transcript from three coding  
574 exons containing respectively the signal peptide, a conserved peptide that does not look like a  
575 neuropeptide, and DH31. In several species, one or two coding exons that code for alternative  
576 neuropeptides have been inserted between coding exons for the conserved peptide and DH31.  
577 This leads to alternative splicing in which different neuropeptides are produced (Fig. 11). In  
578 *Hycleus*, *Tenebrio* and *Tribolium* at least three different mRNAs are produced enabling  
579 precursors sharing the same N-terminal sequence but that have different C-termini encoding an  
580 Arg-amide, a short Pro-amide and the typical DH31 peptide respectively. In contrast to DH31  
581 itself, that has a very well conserved amino acid sequence, these alternative DH31 gene products  
582 lack well defined consensus sequences and are neither very similar to DH31 (Fig. S25).

583 In *Pogonus* there are two additional exons predicted from the trinity assembly of RNAseq  
584 sequences (Fig. 12). In two other Adephaga species, *i.e.* *Gyrinus marinus* and *Carabus*  
585 *granulatus* the transcriptome assembly sequences corresponding to DH31 sequences lack  
586 sequences homologous to those two exons (GAUY02019591.1; GACW01024447.1).

## 587 DH37-DH47 or CRF-like diuretic hormones

588 The *Tribolium* DH37-47 gene has previously been reported to have three coding exons (Li  
589 et al., 2008), in which the first of those three is alternatively spliced to the second or the third one.  
590 This leads to the production of two CRF-like diuretic hormones, DH37 and DH47 which both  
591 been isolated and sequenced from *Tenebrio*, a species of the same family. Given the sequence  
592 similarity of DH47 (Fig. S26) and DH37 (Fig. S27) it seems likely that the last two exons arose  
593 by exon duplication. This gene structure seems to be common to the Cucujiformia, but in the  
594 other Polyphaga and *Pogonus* there are only two coding exons in which the last codes for a CRF-  
595 like hormone. In *Aethina* the DH37 coding exon has been duplicated once more, such that there  
596 are four coding exons in total and three different mRNAs are produced, each encoding different  
597 CRF-like peptides (Fig. 12).

598 Periviscerokinin (Capa)

599 The periviscerokinin genes are quite variable in Coleoptera. They can consist of several  
600 coding exons that all use phase 1 introns. This allows alternative splicing to produce a variety of  
601 different precursors from these genes. Although in some species RNAseq data confirm such  
602 alternative splicing, in many cases the number of total RNAseq reads for these genes is far too  
603 small to demonstrate alternative splicing. An important site of periviscerokinin synthesis is in the  
604 abdominal ganglia, from which RNAseq reads are generally only obtained when whole insects  
605 are used for RNA extraction. Thus while in many species only a single transcript can be  
606 documented, alternative splicing may well be common.

607 The number of coding exons for this gene in the species studied varies between four and  
608 seven (Fig. 13). The first coding exon contains the sequence for the signal peptide, the last for a  
609 hydrophilic C-terminal sequence of the precursor that is usually rich in acidic amino acid  
610 residues. The penultimate coding exon tends to be the largest and codes for subsequently a  
611 periviscerokinin, a tryptopyrokinin and a hydrophobic sequence. The variable number, one to  
612 four exons between the first and penultimate coding exon, contain sequences for a pyrokinin-like  
613 peptide, although in *Hypothenemus*, the third one has lost this sequence.

614 sNPF

615 The sNPF precursor is very well conserved in Coleoptera (Fig. S9). In *Anoplophora* and  
616 *Leptinotarsa*, but not in closely related *Dendroctonus* and *Hypothenemus* or any of the other  
617 species studied here, partial duplication of the exon coding sNPF led to a gene having an  
618 additional coding exon. In *Anoplophora* the RNAseq data suggest the production of two  
619 alternatively spliced transcripts that code for either one or two sNPF paracopies. Although there  
620 is much more RNAseq data from *Leptinotarsa*, in this species there is only evidence for a single  
621 mRNA encoding two sNPF paracopies (Fig. 14).

## 622 **Paracopy numbers**

623 Several insect neuropeptide precursors contain multiple copies of identical or very similar  
624 peptides. These typically include allatostatins A and B, calcitonin B, leucokinin, FMRFamide,  
625 pyrokinin, periviscerokinin, ETH, orcokinin A and B, RYamide, sulfakinin and tachykinins. The  
626 number of such paracopies can vary between and even within species (*e.g.* Veenstra, 2010). The  
627 genes coding calcitonin B, leucokinin, pyrokinin and periviscerokinin have already been  
628 discussed above. Allatostatin A has so far never been found in Coleoptera. ETH has usually two  
629 paracopies, but in the three species from the Elateroidea, *i.e.* *Ignelater*, *Photinus* and *Aquatica*,  
630 the first copy has been lost (Fig. S28) and this is also the case in *Aleochara* and *Hypothenemus*.  
631 In all five of these species, the genome still contains coding sequences for both splice variants of  
632 the ETH receptor. The RYamide gene codes for two RYamide peptides in all Coleoptera (Fig.  
633 S29), except *Anoplophora* that lost this gene and its receptor, while the sulfakinin gene codes also  
634 for two paracopies in all species studied here (Fig S30). The number of FMRFamide paracopies  
635 varies from four to six (Fig. S31), and from 5 to 9 NPLP1 precursor (Fig. S32) and for  
636 allatostatin B (Fig. S33) the numbers are from seven for the Curculionids *Dendroctonus* and  
637 *Hypothenemus* to eight for the other species.

638 Tachykinin

639 The calcitonin B and tachykinin genes are those that show significant changes in the  
640 number of neuropeptides encoded. The ancestral tachykinin gene in Coleoptera likely coded for  
641 eight paracopies, the number found in the majority of species. *Oryctes* and *Harmonia* each lost  
642 one paracopy, but in *Anoplophora* there are only five paracopies and in *Leptinotarsa* there are  
643 just two left. In the latter species, the well conserved N-terminus of the precursor has also  
644 disappeared. This may well be a general phenomenon in Chrysomelidae as the transcriptome  
645 from *Oreina cacaliae* (GDPL01001642.1) reveals a very similar sequence (Fig. S34).  
646 *Leptinotarsa* does have an ortholog of the tachykinin receptor gene that looks normal.

### 647 **Genes that seem very stable**

648 It is fair to state that the number of changes in neuropeptide genes in Coleoptera is  
649 significant. This might obscure the fact that many other genes seem, as least as far as their  
650 sequences are concerned, remarkably stable, such is the case for CCAP (Fig. S35), SIFamide  
651 (Fig. S36), Sulfakinin (Fig. S30), GPA2 (Fig. S37), GPB5 (Fig. S38), FMRFamide, (Fig. S31)  
652 hansolin ( Fig. S39; Liessem et al., 2018), CNMamide (Fig. S40), ITG (Fig. S41) and PTTH (Fig.  
653 S42). The mRNA from the gene coding ion transport peptide (ITP) is generally alternatively  
654 spliced in two forms, ITP-A (Fig. S43) and ITP-B (Fig. S44). It has been reported that in  
655 *Tribolium* there is a third splice product (Begum et al., 2008), but such a form could not be  
656 detected for any of the other species studied here, including *Hycleus* or *Tenebrio*, two species  
657 closely related to *Tenebrio*.

658

### 659 **Discussion**

660 This is the first time that the neuropeptidomes of several species of the same insect order  
661 that are not closely related have been compared. The results clearly show considerable variation  
662 within Coleoptera, variation that seems to be almost as large as that seen between species from  
663 different orders. By using a variety of species some surprising findings, such as *e.g.* the very  
664 evolved structures of NPF and PDF or the loss of certain neuropeptide genes, are confirmed in  
665 related species, and they can thus not be attributed to experimental error.

666 In the same way that there are differences between the different neuropeptides, there are  
667 also differences between the different species. *Leptinotarsa* is perhaps the species in which the  
668 neuropeptidome has evolved the most. It has two vasopressin genes, its allatotropin and sNPF  
669 genes encode two paracopies each, it lost both elevenin and ACP and it duplicated the CCHamide  
670 1 receptor and CCHamide 2 neuropeptide genes. *Anoplophora* is another member of the  
671 Chrysomeloidea superfamily with a neuropeptidome with significant changes. Although  
672 *Anoplophora* still has elevenin and ACP, it lost RYamide and it has a large number of insulin  
673 genes. Both these species are specialist herbivores, like many Cucujiformia. Always eating the  
674 same or almost the same food might eliminate some physiological uncertainties that no longer  
675 need to be regulated. Variation in protein, carbohydrate and water content in food should be  
676 much more limited in specialists than in generalists. For example, if RYamide is indeed an  
677 antidiuretic hormone as suggested (Veenstra and Khammassi, 2016) it might be become obsolete  
678 in a species that is always exposed to the necessity to conserve water. It will no longer be



679 necessary to increase water conservation during times of water shortage and decrease it when the  
680 insect is fully hydrated and thus there may be no longer a need for the acute regulation of  
681 antidiuresis; it always has to be optimal.

### 682 **Significant peptide sequence changes**

683 In those Coleoptera where elevenin and ACP are (still) present neither the sequences of  
684 the peptides nor of those of their precursors are well conserved. Other neuropeptides have not  
685 only maintained the sequences of the neuropeptides themselves, but often those of the entire  
686 precursors, suggesting that those parts of the precursor that do not code for the biologically active  
687 peptides must have other important functions. It has previously been noted that as expression of  
688 the RYamide gene in *Drosophila* species decreases, the structure of both the neuropeptides  
689 themselves and their precursors are no longer well conserved (Veenstra and Khammassi, 2016).  
690 This could mean that the function of the peptide is becoming obsolete, which would facilitate its  
691 subsequent loss; use it or lose it. However, it is also possible that is no longer needed in the  
692 large quantities that are necessary for discharge into the hemolymph. This might well be the case  
693 for *Drosophila* RYamide where the rectal papillae in *D. melanogaster* are innervated by  
694 RYamide neurons. While the same neurons are present in *D. virilis*, in that species – and many  
695 others – RYamide is also released from enteroendocrine cells, presumably likewise to stimulate  
696 the rectal papillae. The amount of RYamide that needs to be released from the midgut to reach  
697 sufficiently high hemolymph concentrations will be much larger than that made by the neurons  
698 that directly innervate the rectal papillae. This likely not only puts selection pressure on the  
699 peptide sequences but also on an efficient processing of their precursors. It is the latter that may  
700 explain why some insect neuropeptide precursors seem to be so well conserved. In *Rhodnius*  
701 and *Tribolium* ACP appears to be expressed exclusively in neurons (Hansen et al., 2010; Patel et  
702 al., 2013). This suggests that its large structural variability indicates a loss of physiological  
703 relevance in Coleoptera which may explain its loss from the genome on at least three occasions in  
704 this insect order. The same could also be true also for elevenin and it is tempting to speculate that  
705 when neuropeptide structures are no longer well conserved it either indicates the loss of their  
706 physiological relevance as a hormone or as both a hormone and a neuromodulator.

707 It is interesting to see that some changes in Coleoptera neuropeptide precursors are similar  
708 to the those observed in other Holometabola. The allatotropin genes in Hemimetabola code for a  
709 single allatotropin paracopy, but in Lepidoptera the gene encodes various allatotropin-like  
710 peptides produced on alternatively spliced mRNAs (e.g. Taylor, Bhatt & Horodyski, 1996;  
711 Nagata et al., 2012). Whereas, the *Pogonus* allatotropin gene is quite similar to those of the  
712 Hemimetabola, in the Polyphaga suborder allatotropin genes coding for two paracopies emerged  
713 on two occasions. The sNPF gene in Hemimetabola is also very simple, but in Lepidoptera and  
714 Diptera, the gene codes for several paracopies. Again this evolved independently in *Anoplophora*  
715 and *Leptinotarsa*. If proctolin is indeed absent from *Oryctes* and the Coccinellids, this would be  
716 similar to what occurred in Hymenoptera and Lepidoptera. Thus in at least some cases  
717 neuropeptide evolution in different holometabolous insect orders seems to follow what look to be  
718 similar pathways, i.e. increasing paracopies in neuropeptide genes that look like they never  
719 changed from the ancestral arthropod to cockroaches, decapods and chelicerates, or

720 independently eliminating others, such as elevenin and ACP. This raises the question whether  
721 somehow complete metamorphosis is responsible for these changes.

722 Several neuropeptides contain a cysteine bridge structure constraining the structure of the  
723 peptide. This could have important effects on receptor binding and/or provide it protection  
724 against proteases degradation. It is surprising to see NPF gain a cysteine bridge in the  
725 Cucujiformia and some, but not all, calcitonins in *Leptinotarsa* and *Anoplophora* lose theirs. It  
726 would be very interesting to see the interactions of these peptides with their receptors in order to  
727 know what effects, if any, these structures have on receptor activation.

## 728 **Gene losses**

729 It appears that loss of a neuropeptide systems is not a very rare event and some  
730 neuropeptides are more easily eliminated than others. Thus, in Coleoptera some neuropeptides  
731 got lost repeatedly, *i.e.* elevenin and ACP each at least three times, natalisin twice and the dilp8  
732 ortholog likely four times. Indeed, elevenin, ACP, calcitonin, corazonin, natalisin, dilp8 and  
733 relaxin were also found missing in other insect species.

734 Although the loss of a neuropeptide signaling system may well have its origin in the  
735 degeneration of the peptide gene, for the reasons of gene sizes, it is as likely to start in the  
736 receptor gene. Not only are the receptor coding regions generally much larger than those of  
737 neuropeptides, but more often than not the total size of these genes is enormous. Thus the  
738 accidental elimination of a large piece of DNA may well be limited to sequences coding a  
739 receptor without altering any adjacent genes, while the elimination of a piece of the same size  
740 that touches a neuropeptide gene is more likely to affect also neighboring genes, thus increasing  
741 the likelihood of selection against such an event. Indeed in at least some instances, one can still  
742 find remnants of the ligand gene, while the receptor has vanished. Apart from calcitonin B in  
743 *Photinus* described here, the same phenomenon is observed with relaxin in *Sitophilus* and  
744 sulfakinin in the tsetse fly *Glossina morsitans*. In the latter species a highly degraded sulfakinin  
745 pseudogene is still recognizable, while both sulfakinin receptor genes have been lost (Unpubl.  
746 Data).

747 As discussed above if the sequence of a neuropeptide is no longer maintained it may  
748 indicate the loss of physiological relevance. This might be a useful criterium to look at duplicated  
749 neuropeptide genes as well. When the putative Coleopteran AKH precursors are compared it is  
750 evident that not all these precursors are well conserved. If the bulk of AKH precursor sequences  
751 is so well conserved, why are the others not? We have a good idea about what signal peptides  
752 and authentic AKHs should look like, and we can thus discard the truly aberrant genes from  
753 *Aethina* and *Harmonia* as obviously no longer functional AKH genes (Fig. 7). However, we do  
754 not know what the requirements are for a good Cys-peptide, as its function is unknown. Similar  
755 Cys-peptides have been found in other insect neuropeptide precursors, such as those of SIFamide  
756 and RYamide (Verleyen, Huybrechts & Schoofs, 2009; Veenstra & Khammassi, 2017). The  
757 conservation of the structure of such peptides implies that they are important – perhaps for  
758 assuring correct intracellular transport to the secretory granules of the neuropeptide precursors –  
759 and thus that those precursors that no longer have such a conserved Cys-peptide may be  
760 functionally impaired. Predicted AKH precursors that look like they might be defective are only



761 found in species that also have an AKH gene with a well conserved AKH precursor. This  
762 suggests that AKH precursors that are not well conserved may have largely lost their functional  
763 significance and/or may be evolving into pseudogenes. It is interesting in this context that of the  
764 two *Tribolium* AKHs only the one that has the best conserved precursor sequence could be  
765 detected by mass spectrometry (Li et al., 2008). Similar arguments suggests that the copy of the  
766 *Oryctes Bursicon A* gene may well be on its way to become a pseudogene.

767 The predicted signal peptides of the proctolin precursors from *Oryctes*, *Harmonia* and  
768 *Coccinella* seem to be perfectly normal as do other parts of the precursor that have been  
769 conserved since the last common ancestor of chelicerates and mandibulates. However, the  
770 predicted proctolin molecules have been mutated and these species all seem to have lost their  
771 proctolin receptors. It is a very puzzling and unresolved matter; as if proctolin is not the only  
772 biologically active peptide produced from the proctolin precursor or as if there is yet another  
773 proctolin receptor that remains to be identified.

774

775

## 776 **Gene duplications**

777 Gene duplications are a common phenomenon during evolution and most of these  
778 duplicated genes are subsequently lost (Lynch, 2007). Genes coding insulin-related peptides and  
779 adipokinetic hormones are repeatedly amplified in insects and in Coleoptera this also includes  
780 pyrokinin genes. Why is it that some neuropeptide genes regularly show increased numbers while  
781 others do so only rarely? When there are paralogous genes in a genome, this is the result of two  
782 independent processes, first duplication of the original gene and then maintaining both the  
783 original and its copy.

784 Just like elimination of receptor gene is likely facilitated by its large size, the initial  
785 duplication of a neuropeptide gene is probably more easily accomplished due to the smaller the  
786 sizes of the gene. It is striking in this context that both the insect AKH and insulin genes – two  
787 that are commonly amplified in insects – are generally very compact genes, have small introns  
788 and plausibly small regulatory regions (of that we only have some information from *Drosophila*,  
789 which is not necessarily a model for all insect species). The Coleoptera pyrokinin genes similarly  
790 look very compact, as the sizes of the introns between the coding regions are small. This is also  
791 true for the strongly amplified vasopressin genes in *Locusta* (Veenstra, 2014).

792 Such small sizes may not only favor the original duplication, but also make it much more  
793 difficult to eliminate the gene by gross chromosome reorganizations and this may explain the  
794 presence of amplified genes in a genome that are not as well conserved as others. However, in  
795 order to permanently maintain two paralog genes, there also needs to be an advantage to  
796 maintaining both copies. This is often achieved through neo- or subspecialization (Lynch, 2007).  
797 In *Drosophila* the different insulin genes do not all have the same temporal and spatial expression  
798 (e.g. Brogiolo et al., 2001; Liu et al., 2016), suggesting that subspecialization may be at least part  
799 of the reason these genes are maintained. I have previously suggested that in some cases it may  
800 be the need for massive amounts of neuropeptides that facilitates the maintenance of paralog

801 neuropeptide genes (Veenstra, 2014). In the case of the Coleoptera pyrokinin genes this may well  
802 apply also, but there is maybe something else at play as well.

803 Pyrokinins, tryptopyrokinins and periviscerokinins are structurally similar arthropod  
804 peptides that each act on specific receptors (Iversen et al., 2002; Rosenkilde et al., 2003;  
805 Cazzamali et al., 2005; Homma et al., 2006; Paluzzi et al., 2010; Paluzzi and O'Donnell, 2012;  
806 Jiang et al., 2014). The tryptopyrokinins are only present in insects, where they seem to play an  
807 important physiological role and they are absent from basal arthropods. In most insect species  
808 they are coded by two different genes, the pyrokinin and periviscerokinin genes. The pyrokinin  
809 genes codes for pyrokinins, often also for a tryptopyrokinin and rarely even a periviscerokinin.  
810 The periviscerokinin gene codes for periviscerokinins, often a tryptopyrokinin and rarely a  
811 pyrokinin. What ever their exact roles or physiological functions, there appears to be a  
812 physiological need to be able to produce these three types of peptides, pyrokinins,  
813 tryptopyrokinins and periviscerokinins independently from one another. In termites, crickets,  
814 stick insects, locusts and cockroaches separate tryptopyrokinin genes have evolved that code for a  
815 tryptopyrokinin precursors containing multiple tryptopyrokinins. However, this has not happened  
816 in holometabolous insects.

817 The tryptopyrokinins are produced predominantly, if not exclusively, by neuroendocrine  
818 cells in the labial neuromere of the suboesophageal ganglion from either a pyrokinin or  
819 periviscerokinin precursor by mechanisms that are not understood. Although receptor ligand  
820 interactions in *Tribolium* suggests that at least in that species there may be no tryptopyrokinin  
821 specific receptor (Jiang et al., 2014), in the closely related *Zoophobas atratus* the periviscerokinin  
822 precursor is still differentially processed to produce predominantly a tryptopyrokinin and  
823 pyrokinin in the suboesophageal ganglion and periviscerokinins in the abdominal ganglia  
824 (Neupert et al., 2018). Interestingly, in *Tribolium* the tryptopyrokinin from the pyrokinin  
825 precursor is less active on the pyrokinin receptors than the one from the periviscerokinin  
826 precursor (Jiang et al., 2014) and so it maybe no coincidence that the tryptopyrokinin from was  
827 lost from a number of Coleoptera pyrokinin genes.

828 Whereas the pyrokinin genes are often amplified in Coleoptera, the periviscerokinin genes  
829 are often sometimes partially amplified, *i.e.* a periviscerokinin coding exon is duplicated. Adding  
830 an extra exon does not change the reading frame, since the introns defining the duplicated exons  
831 are of the same phase. This makes alternative splicing relatively easy. Duplication of this exon  
832 may be further facilitated by the presence of much larger introns than those that are found in the  
833 pyrokinin gene.

834 The size of receptor genes should make their segmental duplication a relatively rare event.  
835 It thus interesting that in *Leptinotarsa* the CCHamide 1 receptor is duplicated and that both  
836 copies seem to be well expressed. Surprising and unexpected is that in the same species the  
837 CCHamide 2 neuropeptide gene is also duplicated. Although this does not constitute final proof  
838 for the evolution of a novel insect neuropeptide system in *Leptinotarsa*, it certainly is as close as  
839 one can get from sequence data alone. Both receptor and peptide have evolved significantly since  
840 their respective duplications (Fig. 8) and this very much suggests that *Leptinotarsa* has three  
841 separate CCHamide neuropeptide systems.

842

**843 Conclusion**

844 Beetle species show very significant differences in their neuropeptidomes. Thus  
845 neuropeptidome variation may be (almost) as big within insect orders as it is between them.  
846

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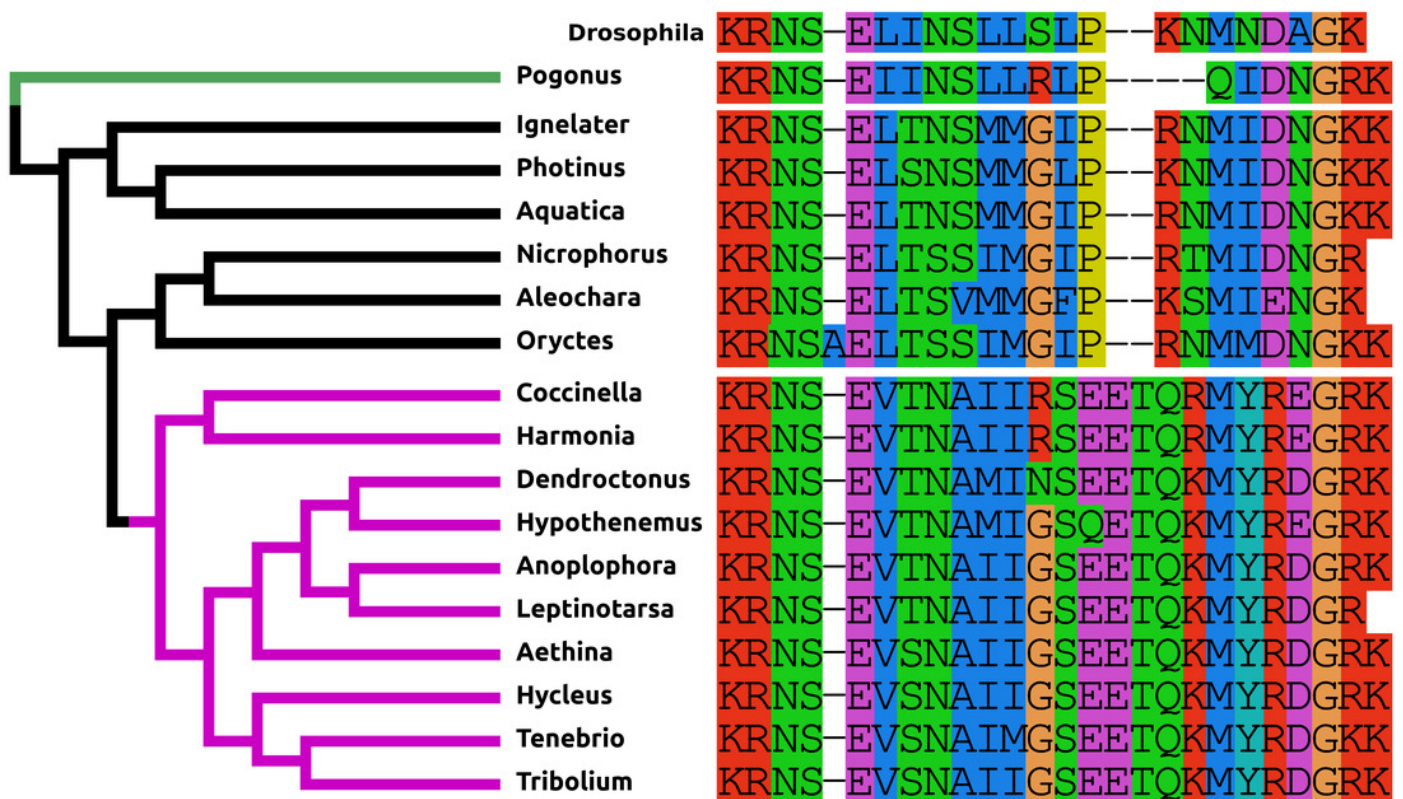
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# Figure 1

## Coleoptera Pigment Dispersing Factors

Alignment of the predicted PDFs from the seventeen Coleoptera species as obtained by conceptual translation of their putative transcripts. *Drosophila* PDF has been added for comparison. The sequences include the processing sites on both side of the mature peptide. The species are arranged according to their position on the phylogenetic tree as established by Zhang and colleagues (2018). Note the differences between the predicted PDF from the single Adephaga species, green on the tree, the Cucujiformia, purple part of the tree, and the remaining Polyphaga species.



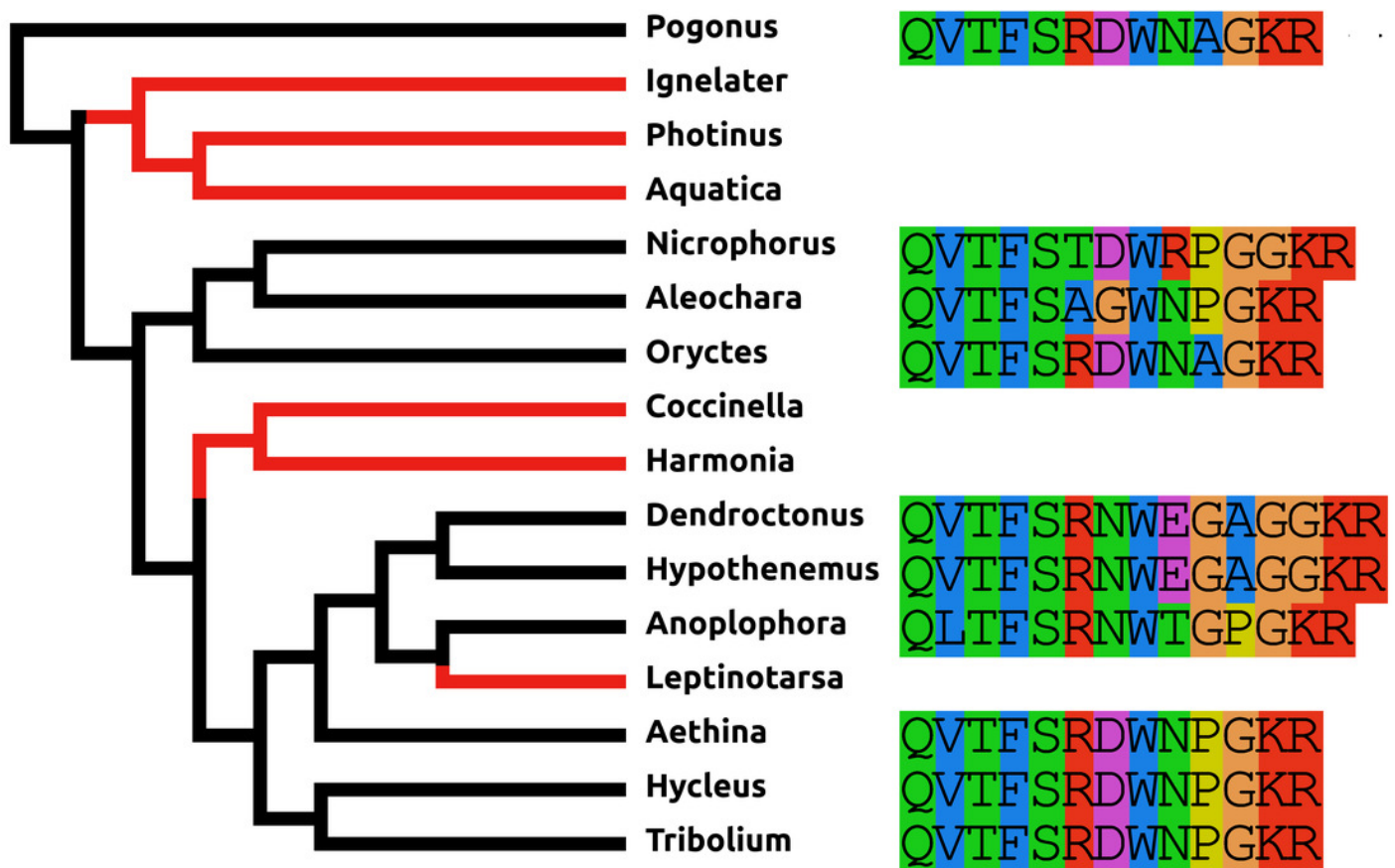




## Figure 3

### Coleoptera ACPs

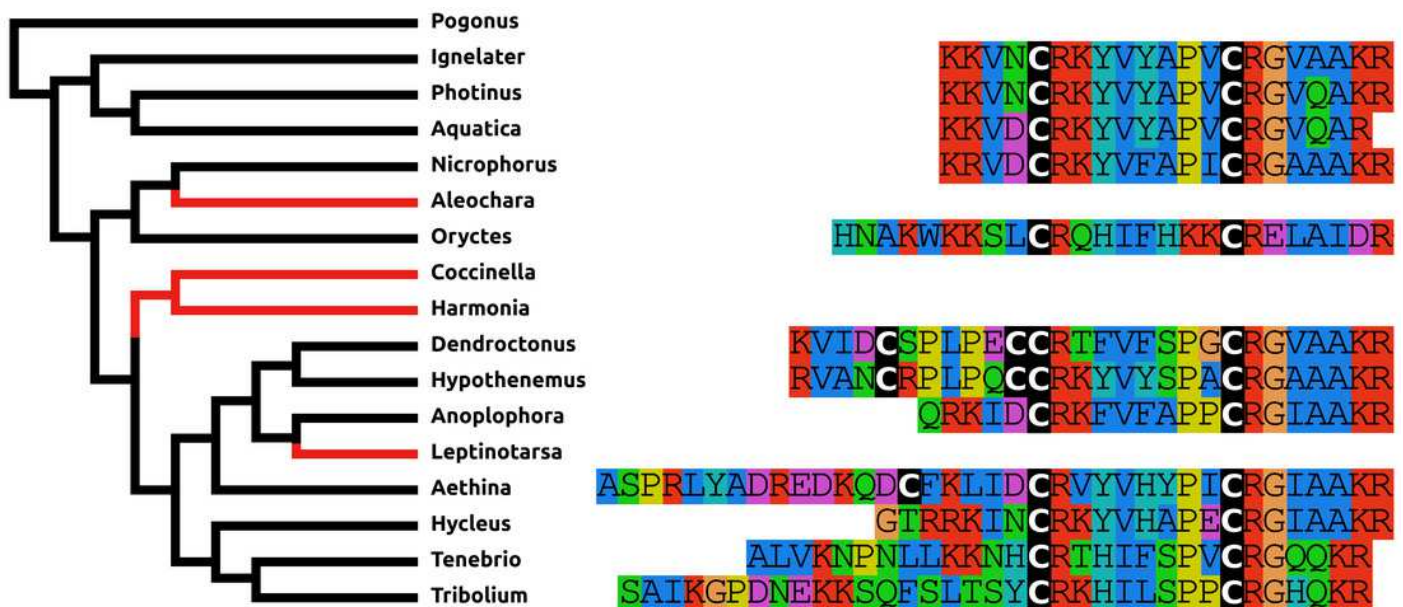
Alignment of the predicted Coleoptera ACPs as obtained by conceptual translation of their putative transcripts. The sequences include the processing sites on C-terminal site of the mature peptide. Cleavage of the N-terminal is performed by a signal peptidase. The species are arranged according to their position on the phylogenetic tree as established by Zhang and colleagues (2018). Tree branches have been made red where the peptide and its receptor were lost from the genome, which must have occurred on at least three occasions. Note that the peptide sequence is not very well conserved.



## Figure 4

### Coleoptera Elevenins.

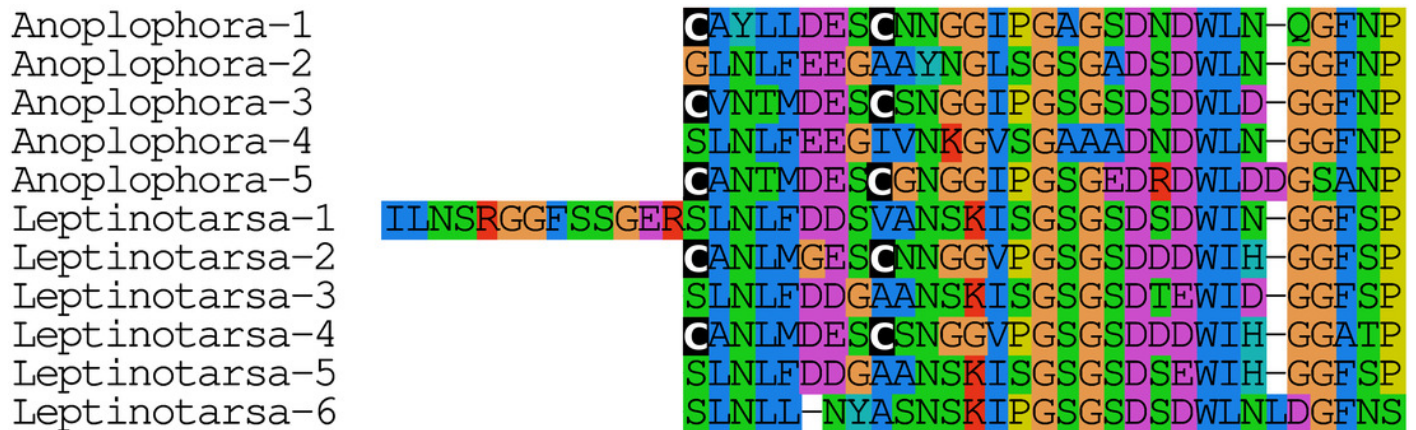
Alignment of the predicted Coleoptera elevenins as obtained by conceptual translation of their putative transcripts. The sequences include the processing sites of peptides; where these are lacking on the N-terminal, cleavage is obtained by a signal peptidase. The species are arranged according to their position on the phylogenetic tree as established by Zhang and colleagues (2018). Tree branches have been made red where the peptide and its receptor were lost from the genome, which must have occurred on at least three occasions. Note that the peptide sequence is not very well conserved and that in both *Dendroctonus* and *Hypothenemus* an additional cysteine bridge has been added to the peptide.



## Figure 5

Unusual calcitonin sequences.

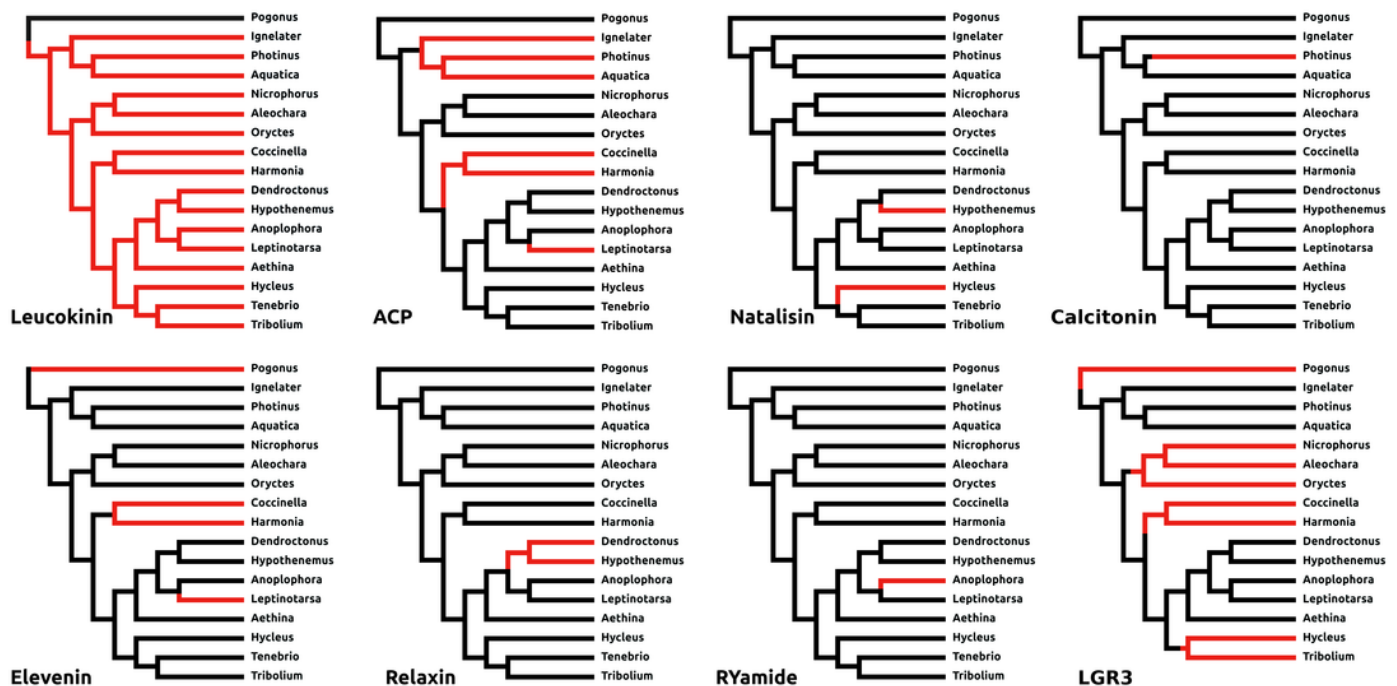
Alignment of calcitonin B sequences encoded by the *Leptinotarsa* calcitonin B gene and the first such gene from *Anoplophora*. Note that with the exception of the sixth peptide from *Leptinotarsa*, these peptides have well conserved amino acid sequences, but that some of them have a cysteine bridge in the N-terminal of the molecule, while others have not. All peptides are predicted to have a C-terminal amide.



## Figure 6

### Neuropeptide losses.

Loss of eight neuropeptide signaling systems in at least one of the sixteen Coleoptera species for which a genome is available. Black branches on the tree indicates the presence of the neuropeptide gene, while red branches indicate its absence. In the case of LGR3 only the receptor could be studied, but for ACP, elevenin, RYamide, natalisin, leucokinin and relaxin both ligand and receptor genes were absent from the indicated genomes. Relaxin and calcitonin are neuropeptides for which the receptor has not been formally deorphanized in insects. Their identities have been deduced from sequence similarity between the insect ligands with their well known vertebrate homologs, sequence similarity between their putative receptors and their vertebrate homologs and the systematic co-occurrence and co-absence in the same genomes of each ligand with its putative receptor (cf Veenstra, 2014).

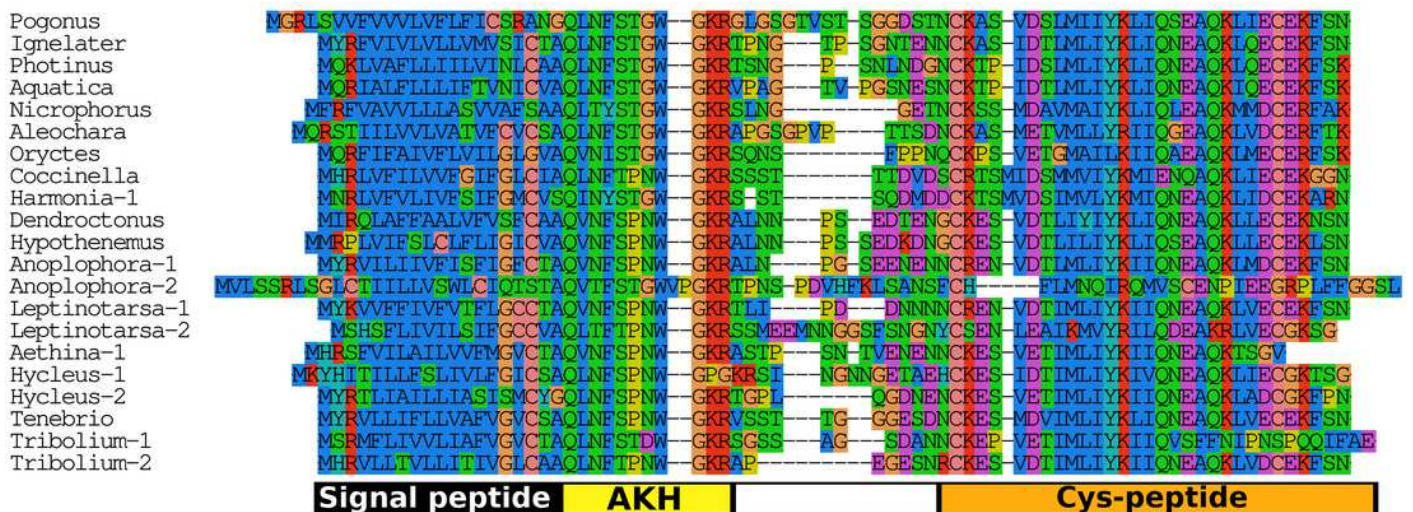




## Figure 7

Adipokinetic hormones.

Putative AKH precursor sequences found in the sixteen genomes and the *Tenebrio* transcriptome. Most of the sequences are relatively short and are aligned in the top part of the figure. Those sequences typically consists of four different parts: the signal peptide, followed immediately by the AKH sequence and a glycine residue that is transformed into the C-terminal amide and a convertase cleavage site, a variable region, and at the end the sequence of a well conserved peptide forming a disulfide bridge. These different regions are indicated below the alignment. Two sequences that show homology to AKH precursors deviate significantly from this pattern. The second *Harmonia* AKH-like precursor is predicted to produce a very long AKH-like peptide, while the second *Aethina* precursor lacks a signal peptide and hence can not produce AKH.



Harmonia-2

MNRFVIVIVAFSIFGICVSQLNFTPYWLIPPEKLIIPROKPMFNSWLOPNAWTNOQPWKRYNSQMDHCKIPMIDSMVVYEMIQEAEKLIIDCEKAKN

Signal peptide AKH Cys-peptide

Aethina-2

MWGFITGWMIHQHINESSEKLIQOTCTELLHSSSSFLALFQLCNSQINFIPNWGKRSPGINDGNNCREPMDSLMVITYKIIQTEAQRMLMCCGKFN

No signal peptide and no AKH Cys-peptide

## Figure 8

*Leptinotarsa* CCHamides.

Duplication of the CCHamide 2 neuropeptide and the CCHamide 1 receptor genes in *Leptinotarsa*. a. Schematic organization of the three CCHamide genes in *Leptinotarsa*. Horizontal lines indicate introns and other untranslated DNA sequences, the boxes correspond to translated exons. Yellow indicates sequences corresponding to the signal peptides, purple correspond to the mature peptide sequences and green the remainder of the precursors. Note that the gene organizations of CCHamides 2a and 2b are very similar. b. Direct comparison of the predicted mature peptides. Note that CCHamide 2b lacks a C-terminal amide that is present in all other CCHamides. c. Direct comparison of the predicted precursors for CCHamides 2a and 2b. Note that, although similar, these sequences are significantly different. d. Simple phylogenetic tree for CCHamide receptors from *Leptinotarsa*, other Coleoptera and *D. melanogaster*. Note that the two *Leptinotarsa* CCHamide 1 receptors are more similar to one another than to any of the other Coleoptera CCHamide 1 receptors, including the one from *Anoplophora*. Nucleotide sequences for these receptors are: ACZ94340.1, XP\_023021283.1; XP\_017768833.1, XP\_019758999.1; XP\_025836439.1, XP\_008197479.1, XP\_023310960.1, XP\_019880954.1; XP\_018332710.1, ERL86066.1, XP\_019880542.1, XP\_023313148.1, XP\_015838444.1; XP\_017779615.1, AAF57819.3, XP\_023023025.1 and the *Leptinotarsa* CCHamide 2 receptor which is present in the supplementary excel file.



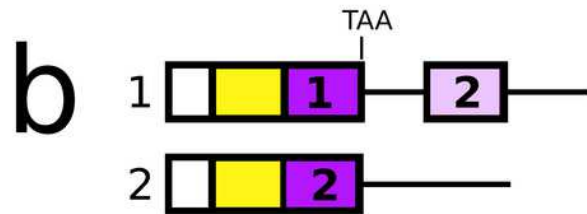
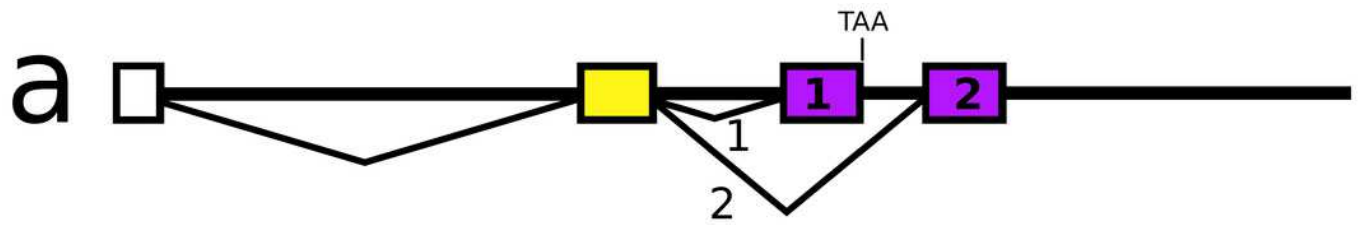


## Figure 9

*Nicrophorus* allatostatin CCC gene.

a. schematic representation of the allatostatin CCC gene in *Nicrophorus*. Boxes indicate exons and horizontal lines introns. The first exon (white) is untranslated, the second (yellow) codes for the signal peptide and few additional amino acid residues. The last coding exon has two acceptor splice sites. b. When the first acceptor splice site is used the mRNA is larger and leads to the production of an mRNA that contains coding sequences for two allatostatin CCC-like peptides, however an inframe stop codon prevents translation of the second one. When the second acceptor splice site is used, it is the second allatostatin CCC peptide that will be produced. c. The last amino acid residues coded by the two types of mRNA.

Convertase cleavage sites and C-terminal dibasic amino acid residues that will be removed by carboxypeptidases are highlighted. The *Aleochara* gene has a very similar structure, although the untranslated first exon could not be identified. Note that the sequences of these peptides are fairly similar between the two species, and that in both cases the peptides produced from the first transcript lack the C-terminal dibasic amino acid residues that are typically present in allatostatin CCC peptides (Veenstra, 2016b).



**C**

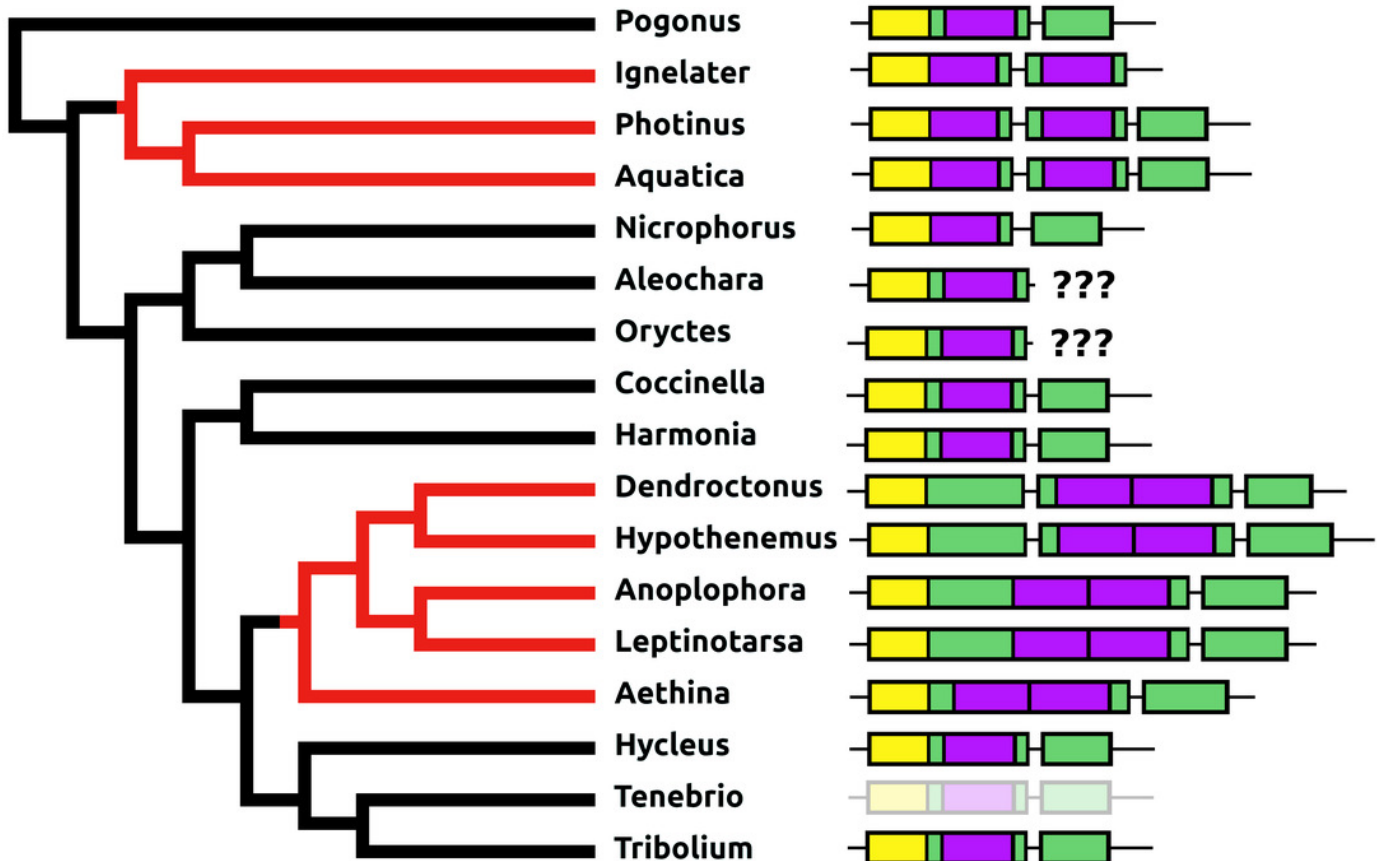
***Nicrophorus***  
 1 KRQTNRLKCYFNPVSCF\*  
 2 KRQSRFRQCYFNPVSCFKK\*

***Aleochara***  
 1 KRQGKRLKCYFNPVSCF\*  
 2 KRASRFRQCYFNPVSCFRK\*

## Figure 10

Structure of allatotropin genes.

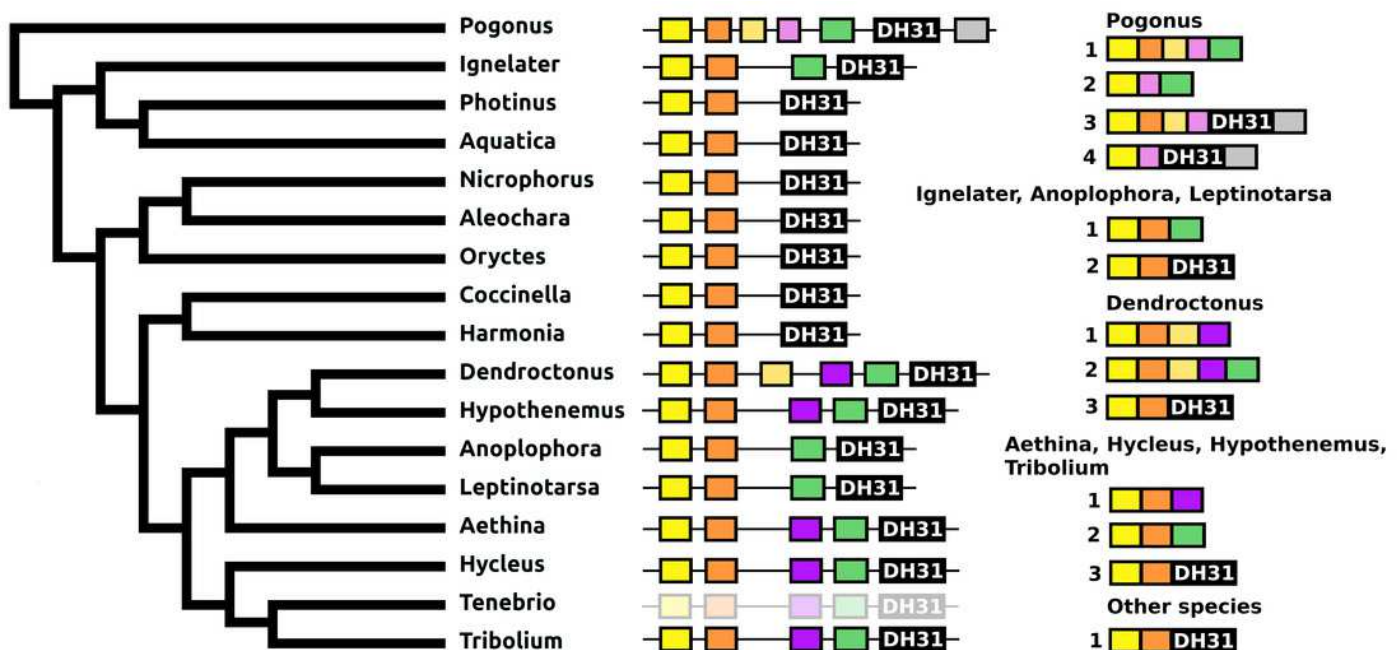
Horizontal lines indicate non-translated DNA and the boxes coding exons. Yellow shows the location of the coding sequences for the signal peptide, purple those for allatotropins and green those for the remainder of the precursors. On two occasions the number of allatotropin paracopies was increased during evolution; red branches in the tree. Once by adding a coding exon, and once by adding a paracopy inside the original allatotropin coding exon. The last coding exons for the *Aleochara* and *Oryctes* allatotropins could not be established. The structure of the *Tenebrio* gene is shaded to indicate that it is only inferred from those of *Hycleus* and *Tribolium* based on the very similar sequences of their respective allatotropin transcripts.



# Figure 11

Structure of DH31 genes.

Horizontal lines indicate non-translated DNA and the boxes coding exons. Yellow shows the location of the coding sequences for the signal peptide coding, orange those for a coding exon common to all transcripts, black coding exons for DH31 itself and green and purple those for other putative neuropeptides. The structure of the *Tenebrio* gene is shaded to indicate that it is only inferred from those of *Hycleus* and *Tribolium* based on the very similar sequences of their respective DH31 transcripts. To the left is a phylogenetic tree in order to facilitate comparing sequences with evolution, to the right are the various transcripts that are produced from these genes by alternative splicing.

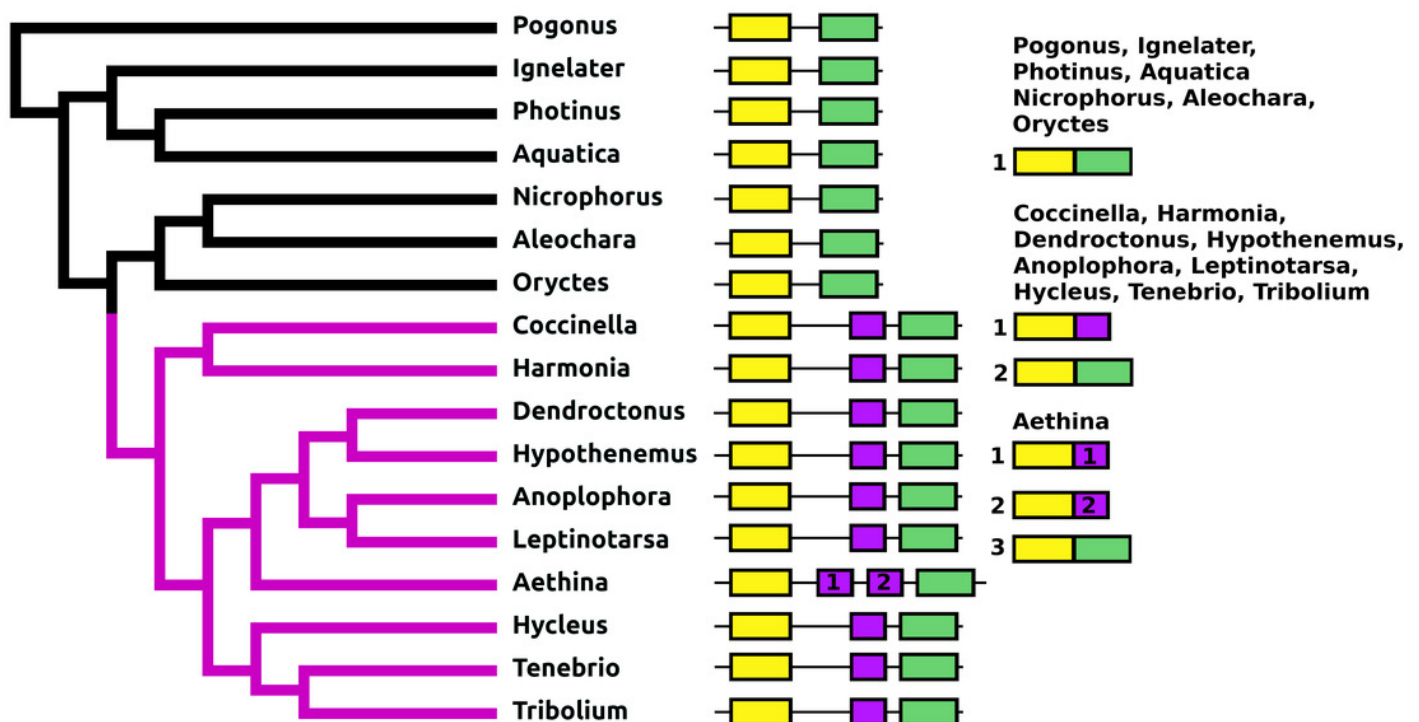




## Figure 12

Structure of D37-D47 genes.

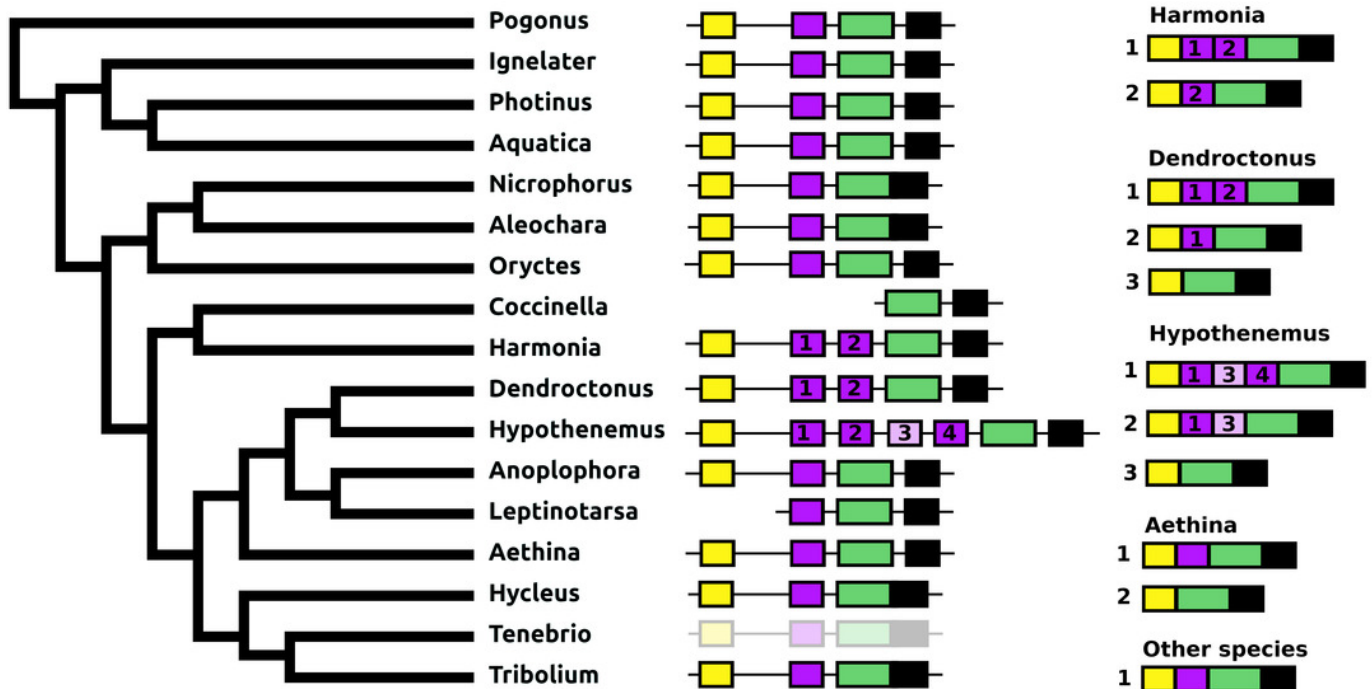
Horizontal lines indicate non-translated DNA and the boxes coding exons. Yellow shows the first coding exon containing sequences coding the signal peptide and parts of the precursor, the purple coding exon contains the complete sequence for DH37, its convertase cleavage sites and few additional amino acid residues on each site, and the green coding exon contains the same for DH47. Note that the DH37 exon is only present in the Cucujiformia, corresponding to the magenta part of the tree. The DH37 coding exon has been duplicated in *Aethina* and allows the DH37-DH47 gene to produce three different transcripts and three different putative diuretic hormones. The structure of the *Tenebrio* gene is shaded to indicate that it is only inferred from those of *Hycleus* and *Tribolium* based on the very similar sequences of their respective DH37 and DH47 transcripts. To the right are the various transcripts that are produced from these genes by alternative splicing.



## Figure 13

Structure of periviscerokinin genes.

Horizontal lines indicate non-translated DNA and the boxes coding exons. Yellow shows the first coding exon containing the sequence coding the signal peptide and parts of the precursor, the purple coding exons contain sequences for a periviscerokinin, and the green coding exon sequences for a periviscerokinin, a tryptopyrokinin and another periviscerokinin. The final exon, black in the figure, characteristically codes for several acidic amino acid residues. In general there are relatively few RNAseq reads for this gene and when there are gaps in the genome assembly, as is the case in *Leptinotarsa* and *Harmonia*, it is not possible to reconstruct the complete gene. The structure of the *Tenebrio* gene is shaded to indicate that it is only inferred from those of *Hycleus* and *Tribolium* based on the very similar sequences of their periviscerokinin transcripts. To the right are the various transcripts that are produced from these genes by alternative splicing. Note that there may well be additional transcripts that could not be identified due to the scarcity of RNAseq reads for this gene.



## Figure 14

sNPF precursors.

Partial tree of four species with the structure of their sNPF genes. In all species except *Anoplophora* and *Leptinotarsa* the sNPF gene consists of three coding exons. The first one (yellow) codes coding for the signal peptide and a few additional amino acid residues, the second one (orange) codes for a well conserved sequence and near the end of the exon has the sequence for sNPF (indicated in green), while the last ones codes for a peptide that is not very well conserved. In both *Anoplophora* and *Leptinotarsa* there is an additional exon between the second and third that codes for an additional sNPF paracopy. In *Leptinotarsa*, RNAseq data suggests a single mRNA encompassing all four of these exons in *Leptinotarsa*, but alternative splicing allowing the production of sNPF precursors that have either one or two sNPF paracopies in *Anoplophora*.

