Juvenile hormone resistance gene Methoprene-tolerant controls entry into metamorphosis in the beetle Tribolium castaneum

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Besides being a spectacular developmental process, metamorphosis is key to insect success. Entry into metamorphosis is controlled by juvenile hormone (JH). In larvae, JH prevents pupal and adult morphogenesis, thus keeping the insect in its immature state. How JH signals to preclude metamorphosis is poorly understood, and a JH receptor remains unknown. One candidate for the JH receptor role is the Methoprene-tolerant (Met) Per-Arnt-Sim (PAS) domain protein [also called Resistance to JH, Rst (1)JH], whose loss confers tolerance to JH and its paralogue, methoprene, in the fruit fly Drosophila melanogaster. However, Met deficiency does not affect the larval–pupal transition, possibly because this process does not require JH absence in Drosophila. By contrast, the red flour beetle Tribolium castaneum is sensitive to developmental regulation by JH, thus making an ideal system to examine the role of Met in the anti-metamorphic JH action. Here we show that impaired function of the Met ortholog TcMet renders Tribolium resistant to the effects of ectopic JH and, in a striking contrast to Drosophila, causes early-stage beetle larvae to undergo precocious metamorphosis. This is evident as TcMet-deficient larvae pupate prematurely or develop specific heterochronic phenotypes such as pupal-like cuticular structures, appendages, and compound eyes. Our results demonstrate that TcMet functions in JH response and provide the critical evidence that the putative JH receptor Met mediates the ant metamorphic effect of JH.

insect metamorphosis | postembryonic development | endocrine regulation | bHLH-PAS domain | RNAi

Metamorphosis is a marked change between larval and adult forms that occurs in all winged insects (1, 2). Holometabulous larvae metamorphose into adults by a stage termed the pupa. More than 70 years ago, V. B. Wigglesworth discovered that the entry into metamorphosis depends on juvenile hormone (JH) (3, 4). In larvae, JH prevents the steroid molting hormones (ecdysteroids) from initiating metamorphosis, so that after a molt another larval stage follows (5, 6). Therefore, in most species, supply of JH makes an insect reiterate its juvenile stage, whereas experimental removal of JH causes it to metamorphose prematurely (1, 7–12). An exception from this paradigm is the “higher” (cycorrhaphous) Diptera including the fruit fly, Drosophila melanogaster, where exogenous JH cannot induce extra larval stages (13). In Drosophila, ectopic JH blocks only adult differentiation of the abdominal histoblasts, whereas the rest of the adult body that derives from imaginal discs is insensitive to JH (14–17).

The mode of JH action remains an enigma, because neither its receptor nor a signaling pathway is known (6, 18–20). In 1986, Wilson and Fabian reported Drosophila mutants that survive toxic doses of JH or its mimic methoprene (21). The Methoprene-tolerant (Met) gene [also called Resistance to juvenile hormone, Rst (1)JH] encodes a transcriptional regulator of the basic helix–loop–helix (bHLH)- Per-Arnt-Sim (PAS) domain family (22). Met has been shown to bind JH at physiological concentrations (23, 24) and therefore is a good candidate for the elusive JH receptor (6, 25–27).

However, the critical evidence that Met is required for the ant metamorphic JH function is missing, because Met-null mutants are fully viable (27–29). A likely reason for the lack of an expected developmental phenotype is the weak effect of JH on preadult Drosophila. Another explanation might be a potential functional redundancy between Met and a paralogue Drosophila gene germ-cell expressed (gce) (30, 31), whose protein product can dimerize with Met in a JH-sensitive manner (26).

The beetle Tribolium castaneum shows the classical developmental response to JH, which can cause repetition of juvenile stages (ref. 32 and this work). Moreover, the recently available genome information indicates that the beetle possesses a single ortholog of Drosophila Met and gce. Tribolium therefore offers an ideal opportunity to examine the role of Met in metamorphosis without the complication of redundancy and the atypical JH response. Here, we show that depletion of Met renders Tribolium resistant to JH, and that in beetle larvae it causes premature pupal morphogenesis. These results establish Met as an essential mediator of the ant metamorphic JH signal and support its putative receptor role.

Results
Met Is Conserved and Constitutively Expressed in Tribolium. Based on the T. castaneum genome database and using rapid amplification of CDNA ends, we have isolated a presumably full-length copy of a transcript that encodes 516 amino acids. The deduced protein is identical to a previously annotated Tribolium protein (XM.961449), except that its N-terminal sequence is 69 amino acids shorter than in the GenBank entry. Our ORF starts with the Kozak translation start site consensus ACCAUGG and is preceded by a stop codon. The protein contains the bHLH domain, two PAS domains (A and B), and a PAS C-terminal motif PAC (Fig. L4) that are all conserved with D. melanogaster Met (22), DmGce, and proteins from mosquitoes (31) and the honey bee (see supporting information (SI)). In its bHLH and PAS-B (but not PAS-A and PAC) domains, the Tribolium protein appears more similar to DmGce than DmMet. The number and positions of Tribolium introns are better conserved with those of DmGce and the Aedes aegypti genes than with the single intron in DmMet (Fig. L4 and ref. 31). Similar to mosquitoes and the honey bee, the Tribolium genome apparently
contains only one counterpart of Dmgce and DmMet. Our phylogenetic analysis confirms a previous notion that Dmgce and DmMet are paralogs that arose by a lineage-specific duplication in higher Diptera (31) and shows that the Tribolium gene is a single ortholog of Drosophila gce and Met (see SI). Because of its functional similarity to Met (see below) and in keeping with the literature, we will refer to the Tribolium gene as TcMet.

To find out whether TcMet is transcriptionally regulated in diverse developmental stages, we analyzed its expression by using RT-PCR (Fig. 1B). The transcript was detected in all stages examined: embryos, fifth instar larvae, prepupae (i.e., immobile pharate pupae that displayed a crooked posture), pupae, and adults. Thus, at the level of mRNA TcMet seems to be continuously expressed throughout beetle development.

**TcMet Silencing Confers Insensitivity to Ectopic JH.** To establish whether TcMet mediates JH response in the beetle as Met does in Drosophila, we have tested for resistance against the hormone and its mimic methoprene in Tribolium pupae. It has been well documented that upon exposure to JH analogs, and its mimic methoprene in Drosophila males. Whether TcMet Silencing Confers Insensitivity to Ectopic JH. Konopova and Jindra PNAS

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**Table 1. TcMet RNAi confers resistance to JH-III and methoprene in Tribolium**

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>Treatment*</th>
<th>n</th>
<th>Second pupae</th>
<th>Unclosed adults</th>
<th>Normal adults</th>
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<tbody>
<tr>
<td>egfp</td>
<td>Methoprene (0.3 mM)</td>
<td>20</td>
<td>20</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Methoprene (0.03 mM)</td>
<td>8</td>
<td>8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>JH-III (0.3 mM)</td>
<td>41</td>
<td>38</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
<td>33</td>
<td>—</td>
<td>—</td>
<td>33</td>
</tr>
<tr>
<td>TcMet</td>
<td>Methoprene (0.3 mM)</td>
<td>26</td>
<td>4</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Methoprene (0.03 mM)</td>
<td>6</td>
<td>1</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>JH-III (0.3 mM)</td>
<td>47</td>
<td>1</td>
<td>14</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
<td>11</td>
<td>—</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>None</td>
<td>Geraniol (57 mM)</td>
<td>35</td>
<td>—</td>
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<tr>
<td></td>
<td>Geraniol (0.6 mM)</td>
<td>15</td>
<td>—</td>
<td>15</td>
<td>—</td>
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<tr>
<td></td>
<td>Farnesol (40 mM)</td>
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<td>—</td>
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<td>31</td>
</tr>
<tr>
<td></td>
<td>Farnesol (0.4 mM)</td>
<td>15</td>
<td>—</td>
<td>—</td>
<td>15</td>
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</table>

*Early seventh- or eighth-instar prepupae were injected with dsRNA and allowed to pupate, then, within 0–12 h after ecdysis, briefly dipped in acetone or acetone-diluted compounds.*
to rescue 98% of pupae (n = 47) from the JH-induced reiteration of pupal development. Similar results were obtained with methoprene (Fig. 2 and Table 1). The increased incidence of second pupae in methoprene-treated TcMet(RNAi) animals was likely due to the fact that methoprene is metabolically nondegradable and therefore more potent than JH-III.

Our data clearly demonstrate that TcMet knockdown renders Tribolium pupae insensitive to natural JH as well as to its analog methoprene. This finding suggests a developmental role for TcMet in JH signaling.

**Loss of TcMet Causes Premature Pupation.** We next asked whether TcMet mediates the ant metamorphic function of JH, which is manifested as extraneurallarval stages instead of pupation in T. castaneum (see SI and ref. 32). Conversely, experimental depletion of JH in many insects including beetles (11, 36) induces premature pupation, and exactly this phenotype would be expected upon TcMet knockdown if JH prevented precocious metamorphosis by TcMet. Under our breeding conditions Tribolium larvae normally pass through seven or (less frequently) eight instars before they pupate.

Injection of TcMet dsRNA into larvae at the beginning of the third and fourth instars caused precocious metamorphosis (Fig. 3). Most notably, RNAi triggered during the third instar induced pupation of four of 52 animals at the end of the fifth instar (Fig. 3A, C, and D). Similarly, silencing of TcMet from the beginning of the fourth instar caused 22% of the larvae to pupate early, usually at the end of their sixth instar (Fig. 3A and F). Some of the minute pupae resulting from these experiments could not completely ecydose from their larval cuticles and soon died (Fig. 3C), whereas others survived for several days, forming superficially perfect yet eventually lethal pharate adults that were unable to ecydose (Fig. 3F). When TcMet dsRNA was diluted 2-fold before injection, we occasionally obtained miniature adult beetles from sixth instar pupae (not shown). By contrast to TcMet RNAi, no signs of precocious metamorphosis were seen in controls that had been injected with egfp dsRNA as third to fifth instar larvae (Fig. 3A, B, and E). On average, 91% of these controls developed to adults normally (Fig. 3A), except that relative to noninjected animals, their pupation was often delayed until the eighth instar, presumably in response to the injury. Because the same injury occurred also in the injected TcMet(RNAi) larvae, their pupation might have been early not by one or two but rather by two or three molts. In addition to early metamorphic phenotypes (see also below), TcMet(RNAi) animals at various stages also suffered ecydysis defects (Figs. 2C, 3C, and 4A). Even larvae that were injected early in the final (seventh) instar and that pupated at the right stage died, being unable to shed the larval or pupal cuticles (not shown). Disrupted ecydysis might thus be a second effect of TcMet deficiency.

On average, 46% of larvae that had been injected with TcMet dsRNA in their third and fourth instars, and nearly all of those injected in the fifth instar, were unable to shed the larval cuticle and died as undersized prepupae (pharate pupae) during the next, i.e., the fifth (Figs. 3A and 4) or sixth instar (Fig. 3E). Before death, these animals displayed a wandering-like behavior that is typical of the final larval instar (37), and premature compound eye development became visible underneath their apolysed larval cuticle (Fig. 4A). To provide further evidence that these TcMet(RNAi) animals were true pharate pupae, two of those that had arrested at the fifth instar were removed from the larval cuticle and subjected to scanning electron microscopy. As expected, both of them showed typical pupal hallmarks such as the presence of wings, gin traps, and pupal genital papillae.

### Table 1

<table>
<thead>
<tr>
<th>Injected stage</th>
<th>n</th>
<th>Larval arrest</th>
<th>Prepupal arrest</th>
<th>Pupal arrest</th>
<th>Adults</th>
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<tr>
<td>L3</td>
<td>52</td>
<td>5 18 1 1</td>
<td>11 5 - -</td>
<td>4 1 2 1</td>
<td>3</td>
</tr>
<tr>
<td>L4</td>
<td>73</td>
<td>- 15 - -</td>
<td>8 33 - -</td>
<td>1 1 5 -</td>
<td>7 1</td>
</tr>
<tr>
<td>L5</td>
<td>25</td>
<td>- - 24 -</td>
<td>- 1 -</td>
<td>- - -</td>
<td>-</td>
</tr>
</tbody>
</table>

#### Fig. 3. Silencing of TcMet in Tribolium larvae induces premature metamorphosis. (A) Summary of the fate of animals injected with dsRNA during third to fifth instars (L3–L5). Numbers in gray box indicate how many larval instars (since hatching) occurred either before the animals died or before they reached adulthood. Whereas 7 and 8 represent the normal count of larval instars, the numbers 5 and 6 imply precocious metamorphic development, manifest as prothetelic larvae and therefore more potent than JH-III.

Whereas 7 and 8 represent the normal count of larval instars, the numbers 5 and 6 imply precocious metamorphic development, manifest as prothetelic larvae and therefore more potent than JH-III.
Fig. 4. Most TcMet(RNAi) larvae are unable to ecdyse and arrest as pre mature prepupa (see Fig. 3A, pink). (A) Arrested fifth instar TcMet(RNAi) prepupa and a control (egfp dsRNA) eighth instar prepupa are shown; both had been injected with dsRNAs as early-third instar larvae. Note the smaller body size and advanced eye pigmentation (arrow) in TcMet(RNAi) prepupa. (B) The prepupa TcMet(RNAi) prepupa from A after it had been removed from the larval cuticle bears typical pupal features including wings (asterisks), gin traps (arrowheads and inset), and pupal (female) genital papillae (arrow). (Scale bars: A, 1 mm; B, 500 μm.)

Thus, up to 67% of the third-to-fifth instar larvae injected with TcMet dsRNA form premature pupae, although most of them fail to ecdyse.

Prothetely Marks Precocious Metamorphosis in TcMet(RNAi) Animals. Approximately 44% and 21%, respectively, of the larvae injected in third and fourth instars arrested after two or three molts (Fig. 3/4). During the instar in which they arrested, the larvae did not feed, sometimes had reduced pigmentation, and finally died within 1 or 2 days after ecdysis, occasionally with pieces of old (previous instar) cuticle attached to their legs or mouthparts. Despite their overall larval appearance, many of these animals displayed a mix of pupal characteristics (prothetely) (Fig. 5). Some had larva-like body only with prematurely advanced development of antennae and eyes (Fig. 5 K and L) or wings of various sizes (Fig. 5M). Other pupal characters in these larvae included gin traps, elongated urogomphi, or pupal genital papillae (compare Fig. 5 N and O with D and I and with E and J, respectively). We also noticed elongated legs with double claws and reduced number of sensillae (not shown). Taken together, our observations reveal that silencing of TcMet in young Tribolium larvae induces precocious pupal morphogenesis.

Discussion

How JH controls metamorphosis at the genetic and molecular level is poorly understood, mainly because neither a JH receptor nor downstream components of JH signaling have been clearly identified. Genetic studies on JH action have been hampered by the fact that the highly evolved Drosophila metamorphosis is only marginally sensitive to JH (16, 17). Thus, although Drosophila has been an excellent system for disclosing important genes such as Met, it has not revealed its role in the fundamental function of JH: maintaining the status quo in juvenile insects.

In this study, we show that Tribolium Met mediates JH response and is required in the beetle larvae for the proper timing of entry to the metamorphic pupal program. Silencing of TcMet produced two major lines of evidence. First, by making pupae resistant to high doses of JH-III and methoprene, it established that the single Tribolium ortholog of the Met/gce genes has a similar function to Met. The function of gce is presently unknown, and future studies in Drosophila should yield interesting comparisons. Second, deficiency of TcMet in young larvae induced premature pupation and other heterochronic phenotypes that mark precocious metamorphosis. The presence of prothetelic larvae likely reflected temporal differences of diverse tissues in the acquisition of the pupal fate and/or their uneven sensitivity to TcMet RNAi. Both variables might bear some influence, because pupal characters in these larvae did not appear in an exact sequence, and individual animals displayed different sets of phenotypes. Generally, however, unlike the appendages, eyes, or wings, the thoracic and abdominal cuticle often retained its larval character, suggesting that, as in other insects (38, 39), Tribolium epidermis becomes pupally committed later than these organs.

Premature metamorphosis can be elicited in many insects by removal of the JH-producing corpora allata gland. However, when such ablation is carried out in very young larvae, one or two additional ecdyses are usually necessary before signs of metamorphosis appear (2, 7, 10). Depletion of JH via continuous overexpression of JH esterase also fails to cause premature pupation in silkworm larvae that are younger than third instar (12), suggesting that insect tissues require a certain time of postembryonic development before acquiring the competence to metamorphose in response to ecdysoid in the absence of JH. These findings help explain why in our experiments metamorphic changes typically followed two or three molts after injection of TcMet dsRNA. Another reason for this delay may be a time lag before sufficient depletion of the protein.

How might TcMet prevent metamorphosis in response to JH? Drosophila Met has been linked with JH in several ways. Met mutants tolerate harmful effects of JH (21, 28), and their tissues display reduced affinity to JH (23). The recombinant Met protein has been shown to bind JH and to stimulate transcription in response to JH-III (24). Finally, Met can form homodimers and heterodimers with Gce in the absence of JH (26). Met thus presents the best known candidate for the JH receptor role. bHLH-PAS domain proteins are particularly interesting signaling molecules, because many of them act as sensors of external stimuli such as hypoxia, xenobiotics or hormones (reviewed in ref. 40). At this point, we have no biochemical evidence for JH binding and JH-dependent transactivation by TcMet; however, our results encourage such studies.

The work presented here discloses the long-elusive role of Met in the JH-controlled process of entry into metamorphosis. This evidence supports the hypothesis that Met might serve as a JH receptor or its essential constituent. Further genetic studies in Tribolium should address interactions of TcMet with other signaling pathways and ultimately improve our understanding of insect metamorphosis and its hormonal regulation.

Methods

Tribolium Breeding and Staging. Wild-type T. castaneum (strain San Bernardino) beetles were maintained on flour–yeast diet at 32°C in constant darkness. Eggs were collected by sifting, and larvae were kept on food in Petri dishes until the desired stage. Because second to final instar larvae cannot be distinguished by obvious morphological markers, staging into instars was based on the number of undergone ecdyses. Under constant condi-
tions, larvae developed at a similar rate, and successive ecdyses thus took place at roughly constant time points since hatching. Therefore, ecdysis into the desired instar was assessed from the timetable of ecdyses that had been prepared as follows: eggs from a 6-h egg collection were placed individually into wells of a cell culture plate and checked daily to mark the time of hatching and of each ecdysis.

Isolation of Tribolium Met cDNA. Conserved region of TcMet spanning the bHLH through PAC domains was identified in the Tribolium genome database (www.bioinformatics.ksu.edu/BeetleBase) by using TBLASTN search with the Drosophila Met protein sequence. A cDNA of 2,710 bp was obtained by using RACE (Invitrogen) from embryonic and larval RNA with two gene-specific primers for 5’/H11032 RACE (5’/H11032-TGGCACTTCTTGCGAC-CATC-3’ and nested 5’/H11032-TGTCCCTTCGCATCTTTTCC-3’/H11032) and two for 3’RACE (5’-ACCACGACCGCCTGGCTATG-3’ and nested 5’-GAAGCTTCAAGAGGAATATG-3’). The full-length transcript was compared with the genome database to identify intron positions.

mRNA Expression Analysis. Total RNA was isolated from dechorionated eggs, whole larvae, pupae, or adults by using TRIzol (Invitrogen, Carlsbad, CA). After treatment with DNase (Roche, Mannheim, Germany), 2 µg of the RNA was used for first-strand cDNA synthesis with oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen). One microliter of 5-fold-diluted cDNA was used for PCR run for 28 cycles with a pair of primers designed for TcMet (5’/H11032-GAAGCTTCAA-GAGAGGAATATG-3’/H11032 and 5’/H11032-TTTCAACAGTTCCCTGGTCG-3’/H11032) and for 24 cycles with a pair of primers for a Tribolium ribosomal protein gene rp49 (5’-TTATGGCAAACT-CAAACGCAAC-3’ and 5’-GGTAGCATGTGCTTCGT-TTTG-3’). That contamination with genomic DNA did not occur was verified by PCR on samples processed without reverse transcriptase. In addition, the primers for TcMet flanked an intron of 4.4 kb.

RNAi. TcMet and egfp dsRNAs, at lengths 564 and 720 bp, respectively, were prepared as described (41) by using the T3 and T7 MEGAscript kit (Ambion, Austin, TX). Larvae and pupae

![Fig. 5. TcMet RNAi causes larval prothetely. TcMet dsRNA was injected at the beginning of the third or fourth larval instar. Shown are examples of heterochronic pupal characters in several different TcMet(RNAi) individuals. When compared with wild-type larvae (A–E) and pupae (F–J), TcMet(RNAi) larvae (K–O) display pupa-like antennae (K, an) and eyes (L). Wings (arrows in H and M) and gin traps (gt) appear in normal pupae and TcMet(RNAi) animals (compare H with M and J with N) but are absent in larvae (see their presumptive positions marked with asterisks in C and D). Urogomphi at the distal abdomen of TcMet(RNAi) animals are elongated like those in pupae (arrows, compare O with E and J). Larval pygopods (E, py) disappear, whereas genital papillae on the ninth abdominal segment develop (arrowheads in J and O, females shown). The larva in A is of the second instar, and larvae in B–E are of the seventh instar. Anterior is to the left in all images; I is a dorsal and N a ventral view. (Scale bars: B, C, G, H, L, and M, 500 µm; D–F and I–K, 200 µm; O, 100 µm; and A and N, 50 µm.)](https://www.pnas.org/)
just after ecdysis were anesthetized with CO2 and injected into abdomens with dsRNA at concentration 5 μg/μl until inflation was visible (42). Insects were then kept individually in 24-well microtiter plates supplied with food and were checked daily to count all ecdyses until a developmental arrest occurred.

**Hormonal Treatments.** Early-seventh or -eighth instar prepupae were injected with TcMet or egfp dsRNA and allowed to pupate. Within 12 h after ecdysis, the pupae were briefly dipped into acetone (control) or acetone-diluted methoprene (VUOS, Par- dubice, Czech Republic), juvenile hormone III, geraniol, or farnesol (the latter three from Sigma, St. Louis, MO).

Scanning-Electron Microscopy. Samples were fixed in 80% ethanol, postfixed with 1% osmium tetroxide, dehydrated in ethanol, critical point dried, gold coated, and observed under a JEOL (Tokyo, Japan) 6500 scanning electron microscope.

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Supporting Results

Supporting Fig. 6

Tribolium 1
Aedes 1
Dm_Gce 1
Apis 1
Dm_Met 1

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Tribolium 53
Aedes 49
Dm_Gce 32
Apis 19
Dm_Met 61

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Tribolium 102
Aedes 98
Dm_Gce 73
Apis 68
Dm_Met 121

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Tribolium 176
Aedes 158
Dm_Gce 130
Apis 126
Dm_Met 181

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Tribolium 201
Aedes 239
Dm_Gce 200
Apis 199
Dm_Met 301

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Tribolium 220
Aedes 276
Dm_Gce 238
Apis 223
Dm_Met 361

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Tribolium 269
Aedes 325
Dm_Gce 287
Apis 273
Dm_Met 421

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Tribolium 329
Aedes 385
Dm_Gce 347
Apis 333
Dm_Met 481

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Supporting Fig. 6. Alignment of *Tribolium castaneum* Met with related proteins from *Aedes aegypti* (EAT47132), *Apis mellifera* (XP_395005) and two *Drosophila melanogaster* proteins, Met (AAC14350) and Gce (NP_511160). The *Tribolium* protein can be accessed under EF468474 or XM_961449 (amino acid residues 70-585). Conserved domains are indicated with lines above the sequence: bHLH (red), PAS-A (green), PAS-B (blue) and PAC (yellow).
Supporting Fig. 7. Phylogenetic analysis. The indicated proteins were aligned by using the ClustalW algorithm implemented in the MEGA 3.1 software (Kumar S, Tamura K, Nei M. 2004. Briefings in Bioinformatics 5:150-163), and the combined conserved domains (outlined in Supporting Fig. 6) were used for construction of phylogenetic trees. (A) Neighbor-joining plot generated by MEGA 3.1 with bootstrapping using 5000 replicates. (B) A plot generated with the Treepuzzle maximum likelihood algorithm (http://bioweb.pasteur.fr/seqanal/interfaces/Puzzle.html) (Schmidt HA, Strimmer K, Vingron M, von Haeseler A. 2002. Bioinformatics 18:502-504) using standard settings. Both trees reveal essentially the same information. The Drosophila melanogaster bHLH-PAS protein Spineless (Ss) (NP_476748) served as an outgroup in both types of analysis.
Supporting Fig. 8. Anti-metamorphic effect of methoprene on Tribolium. Topical application of methoprene at the beginning of the eighth (final) larval instar produced supernumerary larvae. A giant larva that has reached its eleventh instar (i.e., it completed 10 postembryonic ecdyses) is shown on top in comparison with a normal sized eighth instar larva treated only with acetone.