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# Role of the *Halloween* genes, *Spook* and *Phantom* in ecdysteroidogenesis in the desert locust, *Schistocerca gregaria*

Elisabeth Marchal, Liesbeth Badisco, Heleen Verlinden, Tim Vandersmissen, Sofie Van Soest, Pieter Van Wielendaele, Jozef Vanden Broeck *

*Department of Animal Physiology and Neurobiology, Zoological Institute, K.U. Leuven, Naamsestraat 59, B-3000 Leuven, Belgium*

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ABSTRACT

The functional characterization of the *Halloween* genes represented a major breakthrough in the elucidation of the ecdysteroid biosynthetic pathway. These genes encode cytochrome P450 enzymes catalyzing the final steps of ecdysteroid biosynthesis in the dipteran *Drosophila melanogaster* and the Lepidoptera *Manduca sexta* and *Bombyx mori*. This is the first report on the identification of two *Halloween* genes, *spook* (*spo*) and *phantom* (*phm*), from a hemimetabolous orthopteran insect, the desert locust *Schistocerca gregaria*. Using q-RT-PCR, their spatial and temporal transcript profiles were analyzed in both final larval stage and adult locusts. The circulating ecdysteroid titers in the hemolymph were measured and found to correlate well with changes in the temporal transcript profiles of *spo* and *phm*. Moreover, an RNA interference (RNAi)-based approach was employed to study knockdown effects upon silencing of both transcripts in the fifth larval stage. Circulating ecdysteroid levels were found to be significantly reduced upon dsRNA treatment.



## 1. Introduction

The molting hormone was long thought to only be produced by the prothoracic glands (PGs) (or its homologs in Diptera: ring glands) in immature insect stages, where it appeared to be essential for molting and metamorphosis. However, it is now well established that other tissues besides the PGs can perform ecdysteroid synthesis and that the hormone can also play major roles in the reproductive physiology of insects (Simonet et al., 2004; Verlinden et al., 2009). The universal precursor of steroid hormones is cholesterol. In contrast to vertebrate species, insects cannot synthesize cholesterol from simple precursor molecules but need to take it up from their diet. Carnivorous insects can take it directly from their prey, whereas in phytophagous insects, phytosterols are first dealkylated to cholesterol (Gilbert et al., 2002; Gilbert and Warren, 2005). The initial step in 20E biosynthesis is the conversion of cholesterol to 7-dehydrocholesterol (7dC). Between this 7dC and the first upstream compound exhibiting the highly characteristic ecdysteroid structure, diketol, lays the so-called 'Black Box'. This Black Box includes a series of hypothetical and unproven reactions which finally results in the oxidation of 7dC to diketol (Warren et al., 2009). Diketol then undergoes reduction at C3 to form ketodiol (2,22,25-trideoxyecdysone). Next in the pathway, a series of hydroxylation reactions takes place. The genes encoding the enzymes catalyzing these hydroxylations were first identified in *Drosophila melanogaster* using a molecular genetic approach (Gilbert and Warren, 2005). They are members of the *Halloween* class of genes. Mutant embryos for this class of genes share a common phenotype characterized by severe disruptions in morphogenesis, low ecdysteroid titers, undifferentiated cuticle, compact appearance, a failure of head involution and dorsal closure, abnormal looping of the hindgut and inability to induce ecdysteroid responsive genes, eventually followed by death. All these Halloween hydroxylases belong to the family of cytochrome P450 enzymes. It is now known that *phantom* (*phm*, *CYP306A1*) encodes a 25-hydroxylase mediating the conversion of ketodiol to ketotriol (2,22-dideoxyecdysone) (Niwa et al., 2004; Warren et al., 2004); *disembodied* (*dib*, *CYP302A1*) encodes a 22-hydroxylase, catalyzing the reaction from ketotriol to 2-deoxyecdysone (Chavez et al., 2000; Warren et al., 2002); 2-hydroxylase is encoded by *shadow* (*sad*, *CYP315A1*) and converts 2-deoxyecdysone to ecdysone (E) (Warren et al., 2002) and the final hydroxylation from E to the active hormone, 20-hydroxyecdysone (20E) occurs through the action of a 20-hydroxylase encoded by *shade* (*shd*, *CYP314A1*) (Petryk et al., 2003) (Fig. 1). Fully sequenced insect genomes show no paralogs for *phm*, *dib*, *sad* and *shd*. The genes are expressed at all stages of development and moreover, each gene is more similar to their respective orthologs than to any other gene, suggesting that each P450 protein is the sole enzyme catalyzing one specific step in the pathway. This is in contrast with a fifth *Halloween* gene sub-

* Corresponding author. Tel.: +32 16 323978; fax: +32 16 323902.
*E-mail address:* jozef.vandenbroeck@bio.kuleuven.be (J. Vanden Broeck).

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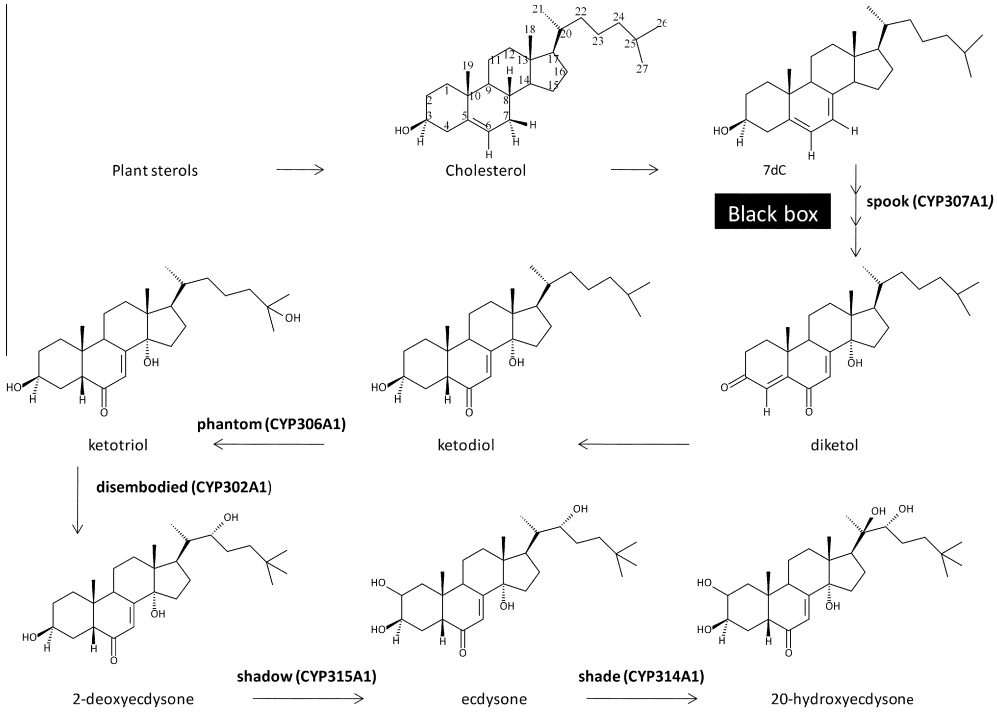


**Fig. 1.** Biosynthetic pathway of 20E in the fly *D. melanogaster* and the different cytochrome P450 enzymes involved. The CYP307 family probably plays a role in the 'Black Box'. CYP306A1, CYP302A1, CYP315A1 and CYP314A1 catalyze the final hydroxylation reactions. Figure based on "Scheme of 20-hydroxyecdysone (20E) biosynthesis in *Drosophila*" (Gilbert and Warren, 2005).

family (*CYP307*) for which several paralogs were found: *spook* (*spo*, *CYP307A1*), *spookier* (*spok*, *CYP307A2*) and *spookiest* (*spot*, *CYP307B1*) (Rewitz et al., 2007). In *Drosophila*, only *spo* (*Cyp307a1*) and *spok* (*Cyp307a2*) are found. CYP307A1 and CYP307A2 probably act in the above described 'Black Box' but their exact biochemical function remains unknown (Ono et al., 2006). Since their first functional identification in *D. melanogaster*, a similar function for these *Halloween* genes has also been described in two lepidopteran species: the silkworm *Bombyx mori* (Maeda et al., 2008; Niwa et al., 2004, 2005; Warren et al., 2004) and the tobacco hornworm, *Manduca sexta* (Rewitz et al., 2006a,b). *Halloween* genes have also been described/predicted in several other Arthropod species: in another lepidopteran, the cotton leafworm, *Spodoptera littoralis* (Iga and Smagghe, 2010), but also in the dipteran *Aedes aegypti* (Sieglaff et al., 2005), the hemipteran pea aphid *Acyrthosiphon pisum* (Christiaens et al., 2010) and the crustacean *Daphnia pulex* (Rewitz and Gilbert, 2008). The description of the *Halloween* genes in these species is mostly based on sequence similarity. The presence of *Halloween* orthologs in the crustacean *Daphnia* suggested that these *Halloween* genes became functionally specialized before the crustacean and insect split and have been under selective pressure ever since. This would imply that orthologs probably exist in the hemimetabolous desert locust as well. However, the functionality of this class of genes within the hemimetabolous insects has never been described so far. This report will be focused on the study of transcript profiles and the RNAi-based characterization of *spo* and *phm* orthologs in a major pest insect, the hemimetabolous desert locust, *Schistocerca gregaria*. These locusts can live scattered as solitarious insects, but can also, under certain environmental conditions, aggregate to form huge swarms that set out in the search of food. Locust swarms evidently pose a major threat to the agricultural production in their habitat, which ranges from West Africa to India and includes some of the world's poorest countries. Their voraciousness, high reproduction rate and migrating behavior make these locusts a very difficult pest to control efficiently. The specificity of these Halloween genes implies that they may constitute possible screening targets for new and more selective pest control compounds. Using q-RT-PCR, a tissue distribution was analyzed for *Sgspo* and *Sgphm* transcripts. Based on the results, a temporal transcript profile was made for both genes in the final larval stage and female adult development in the tissue in which the highest relative mRNA levels were found. These profiles were shown to correlate with the circulating ecdysteroid titer in the hemolymph. Finally, the transcript levels of *Sgspo* and *Sgphm* were downregulated upon injection of dsRNA and the possible effects on the ecdysteroid titers were evaluated.

## 2. Materials and methods

### 2.1. Animals

The desert locusts *S. gregaria* and migratory locusts *Locusta migratoria* were reared under laboratory conditions, under a 13 h light, 11 h dark photoperiod at a constant temperature of 32 °C and ambient relative humidity between 40% and 60%. They were reared in large cages (38 × 38 × 38 cm) under crowded conditions. The animals were fed daily with dry oat flakes and fresh cabbage or grass *ad libitum*. Following mating, mature females deposited their eggs in pots filled with slightly moistened sterile sand. After oviposition, these pots were collected each week and set apart in empty cages, where eggs were allowed to hatch into first instar larvae. In the described experiments, animals were synchronized on the day of ecdysis into the 5th larval stage and dissected every other day during the final instar development. Similarly, adult locusts were synchronized on their day of ecdysis (of the 5th larval to adult stage) and dissected every other day until they were 18 days old. Adult female locusts were not only synchronized at their day of ecdysis but were also staged during dissection according to oocyte length, to make sure female animals within the same pool were