

Supplementary Materials

Table S1. List of primers used to prepare probes for ISH.

The *D. guttifer* *t* exon 5 forward and reverse primer pair was used to amplify *D. guttifer* genomic DNA to produce the probe used to perform the *tan* ISH in *D. palustris* and *D. subpalustris*. The forward and reverse primer pair for *D. guttifer* *y* exon 2 was used to amplify *D. guttifer* genomic DNA to develop the probe to determine *yellow* gene expression in *D. guttifer* and *D. deflecta*. The *D. palustris* forward and reverse primer pair for *y* exon 2 was used to amplify *D. quinaria* and *D. recens* genomic DNA to make the probes used to determine *yellow* gene expression patterns in both *D. recens* and *D. quinaria*. We used the probes generated from a different species' DNA to perform ISH due to the close evolutionary relationships of species within the *quinaria* species group. All internal forward and internal reverse primer pairs were used for verification of the gene identity during the probe-making process.

Primer Name	Primer Sequence
<i>D. guttifer</i> <i>t</i> exon 5 forward	CAGCGTCTGCTTGGCCACACG
<i>D. guttifer</i> <i>t</i> exon 5 reverse	TTGCCGCTGCGCAACAATTCGG
<i>D. guttifer</i> <i>t</i> exon 5 internal forward	GCTGAATCATTACTACTTTGTGG
<i>D. guttifer</i> <i>t</i> exon 5 internal reverse	AATGGTGTTGATGCTGAACACG
<i>D. palustris</i> <i>y</i> exon 2 forward	GAGGAGGGCATCTTTGGC
<i>D. palustris</i> <i>y</i> exon 2 reverse	CGATGCCATGGAATTGCGG
<i>D. palustris</i> <i>y</i> exon 2 internal forward	TCTCGCACCGAGGACAGC
<i>D. palustris</i> <i>y</i> exon 2 internal reverse	CGATCAGATTGAACAGCTCG
<i>D. melanogaster</i> <i>wg</i> exon 4 forward	CACGTCCAAGCGGAGATGCG
<i>D. melanogaster</i> <i>wg</i> exon 4 reverse	GGCGACGGCATGTTCGGGTG
<i>D. melanogaster</i> <i>wg</i> exon 4 internal forward	TGCCATGGCATGTCCGGATCG
<i>D. melanogaster</i> <i>wg</i> exon 4 internal reverse	G TTCAGCATACGCTCCTCCTCC
pGEM®-T Easy M13F	GTAAAACGACGGCCAGT
pGEM®-T Easy M13R	CAGGAAACAGCTATGAC

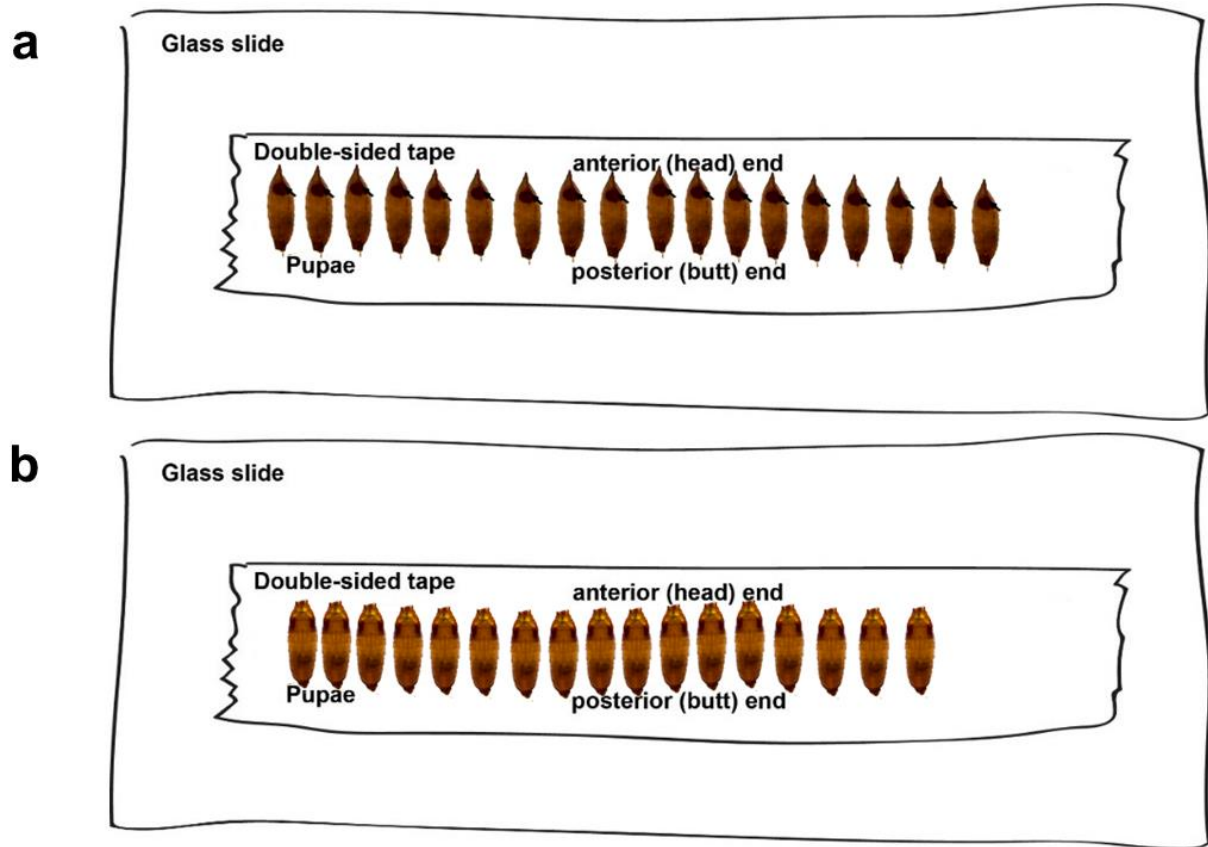


Figure S1. *D. guttifer* pupae lined up for lateral and dorsal cuts. (a) The pupae were positioned on the side to perform a cut that separates the dorsal from the ventral half (lateral cut) (b) the pupae were placed with their ventral side facing the tape to make a cut between both eyes (dorsal cut).

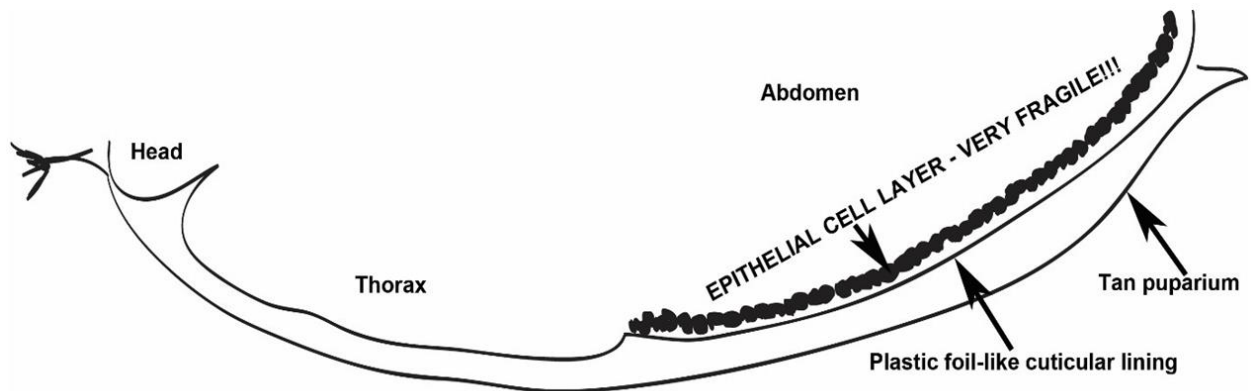


Figure S2. A sketch of a *Drosophila* pupal abdomen showing the internal epithelial cell layer and the cuticular lining holding the cells.

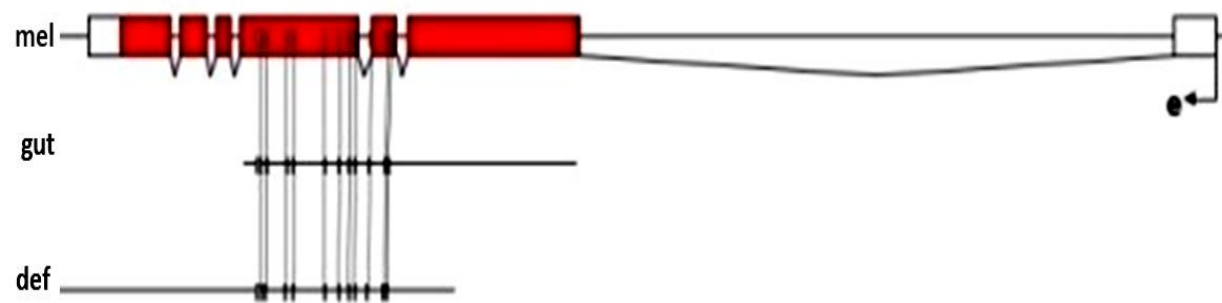


Figure S3. A graphical representation of the conserved region on the *ebony* gene of *D. melanogaster* (top row), *D. guttifera* (gut), and *D. deflecta* (def) [24].