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# Malpighian tubule development in the red flour beetle (*Tribolium castaneum*)

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ABSTRACT

Malpighian tubules (MpTs) are the major organ for excretion and osmoregulation in most insects. MpT development is characterised for *Drosophila melanogaster*, but not other species. We therefore do not know the extent to which the MpT developmental programme is conserved across insects. To redress this we provide a comprehensive description of MpT development in the beetle *Tribolium castaneum* (Coleoptera), a species separated from *Drosophila* by >315 million years. We identify similarities with *Drosophila* MpT development including: 1) the onset of morphological development, beginning when tubules bud from the gut and proliferate to increase organ size. 2) the tubule is shaped by convergent-extension movements and oriented cell divisions. 3) differentiated tip cells activate EGF-signalling in distal MpT cells through the ligand Spitz. 4) MpTs contain two main cell types – principal and stellate cells, differing in morphology and gene expression. We also describe development of the beetle cryptonephridial system, an adaptation for water conservation, which represents a major modification of the MpT ground plan characterised by intimate association between MpTs and rectum. This work establishes a new model to compare MpT development across insects, and provides a framework to help understand how an evolutionary novelty – the cryptonephridial system – arose during organ evolution.

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## 1. Introduction

For most insects the Malpighian tubule (MpT) is the principal organ of excretion. It controls water, ion and acid/base balance, and removes toxins and metabolic wastes. MpTs are simple, single cell-layered epithelial tubes equipped for the transport of water, ions and toxins, most usually from the haemolymph into the tubule lumen. The contents of the lumen are modified before being expelled into the hindgut, where further modification can also take place (Beyenbach et al., 2010; Denholm, 2013).

Although their general form is similar across the insects, MpTs can vary substantially in size and number, ranging for example from the two long pairs of tubules found in *Drosophila* and in the blood-sucking bug *Rhodnius*, to the few hundred shorter tubules found in locusts and crickets (Skaer, 1992; Skaer et al., 1990; Hazelton et al., 1988; Wessing and Eichelberg, 1978; Snodgrass, 1935; Wigglesworth, 1939, 1931). In some species the MpTs have been modified for specialised functions. The rectal complex or cryptonephridial system, which is particularly prevalent in Coleopterans and in the larvae of Lepidopterans, is one example (Fig. 1A) (Ramsay, 1964; Grimstone et al., 1968; Ramsay, 1976; Saini, 1964). Here, the distal portions of the tubules form an intimate association with the rectum in a system that allows water to be drawn osmotically from the contents of the rectum back into the tubule, and from there recycled back to the haemolymph from the proximal tubule. The cryptonephridial system is considered an adaptation to minimize water loss.

*Drosophila melanogaster* stands alone as the one insect in which genetic and molecular development is known in detail. Over the last few decades we have learned how this organ is specified, how it grows and is shaped, and is positioned within the body of the animal (Skaer, 1989; Ainsworth et al., 2000; Kerber et al., 1998; Sudarsan et al., 2002; Denholm et al., 2003; Bunt et al., 2010;

*Abbreviations:* BMP, bone morphogenetic protein; CE, convergent-extension; DAPI, 4′,6-diamidino-2-phenylindole; D-P, disto-proximal; dpERK, di-phosphorylated ERK; Dpp, decapentaplegic; EGF, epidermal growth factor; GFP, green fluorescent protein; MpT, Malpighian tubule; PBS, phosphate buffered saline; PC, principal cell; SC, stellate cell; SD, standard deviation; Spi, Spitz; Tc, *Tribolium castaneum*; TC, tip cell; Tio, Tiptop; Tsh, Teashirt.

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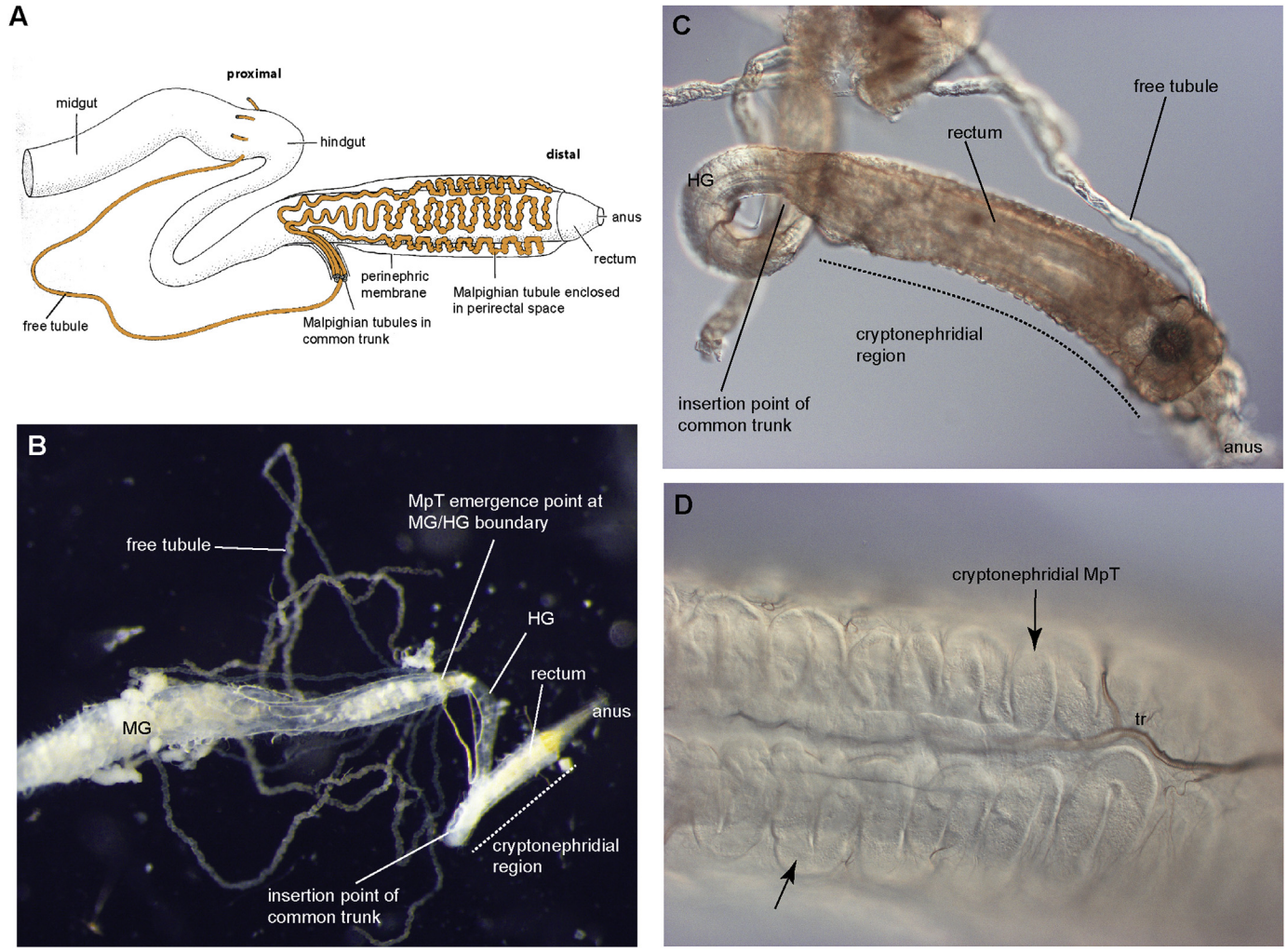


**Fig. 1.** The Malpighian tubules and cryptonephridial system in *Tenebrio* and *Tribolium*. A. Drawing of the Malpighian tubules and cryptonephridial system from an adult mealworm beetle (*Tenebrio molitor*). Image adapted from (Gullan and Cranston, 2010). B. A dissected adult gut from *Tribolium*. Tubules emerge at the midgut (MG)/hindgut (HG) boundary and the free tubules loop anteriorly in the abdomen. The free tubules gather in a common trunk (not visible in figure) and enter the cryptonephridial system (dotted line). C. Detailed view of *Tribolium* larval cryptonephridial system. D. Higher magnification view of image shown in C, cryptonephridial MpTs (arrows) and trachea (tr) are indicated. Anterior in this and all subsequent figures is to the left.

Weavers and Skaer, 2013; Denholm et al., 2005; Hatton-Ellis et al., 2007; Wan et al., 2000; Hoch et al., 1994). We also have an appreciation for the diversity of its cell types (Sozen et al., 1997; Skaer, 1989; O'Donnell et al., 1996, 1998; Dow, 2012; Rosay et al., 1997) and how cellular differentiation is established during development (Denholm et al., 2013). It is not known whether a similar developmental programme is conserved in other insects. Further, the question of how specialised features, such as the cryptonephridial system, emerge during development has not been investigated. For these reasons we have analysed embryonic MpT development from the model beetle species *Tribolium castaneum* (Coleoptera). This work provides a comparative model of insect excretory organ development and morphology, and establishes a framework to help understand how a novel evolutionary adaptation – the cryptonephridial system – came about during evolution.

## 2. Materials and methods

### 2.1. Embryo and tissue processing

Adults were allowed to lay on white flour at 25 °C over a period of a few days. Embryos were harvested by sieving, dechorionated with bleach, washed with water and fixed with shaking in heptane/4% paraformaldehyde (in PBS) for 30 min. They were then transferred to methanol and stored at −20 °C, or processed immediately. To devitellinize, the embryos were decanted onto a small piece of plastic mesh and rinsed once with PBS, transferred onto Scotch double-sided sticky tape on a microscope slide using a paintbrush, and covered with PBS. A PAP pen was used to corral the liquid. Embryos were devitellinized by hand using sharpened tungsten needles. For phalloidin staining freshly fixed embryos were used, with the methanol step omitted.

Adult tubules were dissected in PBS and accumulated in 4% paraformaldehyde (in PBS) on ice, they were fixed for 30 min at room temperature with shaking.

### 2.2. Immunohistochemistry

Antibody staining was carried out under standard conditions. Antibodies used were: rat anti-Tio (1:200) (Laugier et al., 2005); mouse anti-Cut (1:200, DSHB); rabbit anti-phospho histone H3 (1:1000, Upstate Biotechnology); rabbit anti-dpERK (1:50, Cell Signaling Technology); goat anti-GFP (1:500, abcam). FITC- or Cy3-conjugated secondary antibodies were used for fluorescent

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