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SHORT COMMUNICATION

Larval RNAi in *Drosophila*?

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Abstract RNA interference (RNAi) has become a common method of gene knockdown in many model systems. To trigger an RNAi response, double-stranded RNA (dsRNA) must enter the cell. In some organisms such as *Caenorhabditis elegans*, cells can take up dsRNA from the extracellular environment via a cellular uptake mechanism termed systemic RNAi. However, in the fruit fly *Drosophila melanogaster*, it is widely believed that cells are unable to take up dsRNA, although there is little published data to support this claim. In this study, we set out to determine whether this perception has a factual basis. We took advantage of traditional Gal4/upstream activation sequence (UAS) transgenic flies as well as the mosaic analysis with a repressible cell marker (MARCM) system to show that extracellular injection of dsRNA into *Drosophila* larvae cannot trigger RNAi in most *Drosophila* tissues (with the exception of hemocytes). Our results show that this is not due to a lack of RNAi machinery in these tissues as overexpression of dsRNA inside the cells using hairpin RNAs efficiently induces an RNAi response in the same tissues. These results suggest that, while most *Drosophila* tissues indeed lack the ability to uptake dsRNA from the surrounding environment, hemocytes can initiate RNAi in response to extracellular dsRNA. We also examined another insect, the red flour beetle *Tribolium castaneum*, which has been shown to exhibit a robust systemic RNAi response. We show that virtually all *Tribolium* tissues can respond to extracellular dsRNA, which is strikingly different from the situation in *Drosophila*. Our data provide specific information about the tissues amenable to RNAi in two different insects, which may help us understand the molecular basis of systemic RNAi.

Keywords RNAi · Systemic · *Tribolium castaneum* (red flour beetle) · *Drosophila melanogaster* (fruit fly) · Injection

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Introduction

Since its initial characterization in *Caenorhabditis elegans*, RNA interference (RNAi) has become a powerful genetic tool in many organisms, allowing the knockdown of homologous gene products by the introduction of double-stranded RNA (dsRNA) into cells (May and Plasterk 2005). Introduction can be achieved by a variety of methods including microinjection, electroporation, and hairpin RNA expression (May and Plasterk 2005). However, for some organisms, such as *C. elegans*, artificial introduction of dsRNA directly into cells is not required (Fire et al. 1998). In these organisms, the dsRNA is taken up from the extracellular environment (and spread from cell to cell) via a cellular uptake mechanism termed systemic RNAi (Fire et al. 1998).

In recent years, a variety of insects, including the red flour beetle *Tribolium castaneum* (Bucher et al. 2002; Tomoyasu and Denell 2004), have been shown to exhibit systemic uptake of dsRNA (sometimes in limited tissues;

see Tomoyasu et al. 2008 for more details about insect species that show a systemic RNAi response). However, for the well-established insect model system, *Drosophila melanogaster*, there is a widely held belief that its cells are unable to take up dsRNA in a systemic manner. While this perception remains entrenched in the fly community, little empirical data have been published to support this claim. In fact, there are published reports of successful RNAi by injection of dsRNA in *Drosophila* adults (Dzitoyeva et al. 2001; Goto et al. 2003; Petruk et al. 2006).

In this study, we set out to determine whether the belief that *Drosophila* cells are unable to take up dsRNA from their environment holds true. For comparison, we also examined dsRNA uptake in *Tribolium*. We find that systemic RNAi in larval tissues indeed differs drastically between these two insect species. While virtually all larval tissues in *Tribolium* are able to take up dsRNA and mount an RNAi response, only one larval cell type in *Drosophila*, the hemocyte, is able to respond to injected extracellular dsRNAs.

Materials and methods

Fly stocks and crosses

Flies were raised at 25°C. The Gal4 lines used in this study and their expression patterns are summarized in Table 1. These lines were crossed to either *UAS-EGFP/TM3* or *UAS-GFP^{S65T}* to obtain green fluorescent protein (GFP)-expressing larvae for injection. The *UAS-EGFP*-RNAi line [*w; P(UAS-Avic/GFP.dsRNA.R)143* (Roignant et al. 2003)] was also used to induce the RNAi response in these enhanced green fluorescent protein (EGFP)-expressing larvae. *Tub-Gal4; Tub-Gal80* flies [*w; tubP-Gal80; tubP-Gal4/TM6B, Tb* (Lee and Luo 2001)] were crossed with *UAS-GFP^{S65T}* for the Gal80 RNAi experiment.

Beetle strains

Beetles were cultured at 30°C on whole wheat (+5% yeast) flour. The strain AT^{11}, in which EGFP expression is driven ubiquitously by the *aTub* promoter, was used for injection.

dsRNA synthesis

Double-stranded RNA was synthesized using the Ambion MEGAscript high-yield transcription kit. Template for the synthesis of EGFP dsRNA was prepared by polymerase chain reaction (PCR) using gene-specific primers with a T7 polymerase promoter sequence at the 5′ ends as described by Tomoyasu and Denell (2004). Templates for the synthesis of GFP and Gal80 dsRNA were prepared by PCR using vector-specific primers with a T7 polymerase promoter sequence at the 5′ ends as described by Tomoyasu et al. (2008).

Injection into beetle larvae

Injection into beetle larvae was performed as previously described by Tomoyasu and Denell (2004). dsRNA was injected at a concentration of 1 μg/μl (approximately 0.5 μg per larva).

Table 1 Transgenic *Drosophila* lines used in this study

Gal4 Line	Expression	Genotypes for Injection	dsRNA	Hairpin Genotype
w; P{GawB}48Y	Brain salivary glands	*w; P{GawB}48Y/+; P{UAS-EGFP}34/+*	EGFP	*w; P{GawB}48Y/ P{UAS-Avic/GFP. dsRNA.R}143; P{UAS-EGFP}34/+*
y^{1}w; P{en2.4-Gal4}e16EP {UAS-FLP1.D}JD1	Segmental ectoderm	*w; P{en2.4-Gal4}e16EP{UAS-FLP1. D}JD1/+; P{UAS-EGFP}34/+*	EGFP	*w; P{en2.4-Gal4}e16E P{UAS-FLP1.D} JD1/ P{UASAvic/GFP.dsRNA.R}143; P{UAS-EGFP}34/+*
w; P{GawB}c179	Muscles salivary glands	*w; P{GawB}c179/+; P{UAS-EGFP}34/+*	EGFP	*w; P{GawB}c179/ P{UAS-Avic/GFP. dsRNA.R}143; P{UAS-EGFP}34/+*
w^{1118}; P{Cg-GAL4.A}2	Fat bodies	*w; P{Cg-GAL4.A}2/+; P{UAS-EGFP}34/+*	EGFP	*w; P{Cg-GAL4.A}2/ P{UAS-Avic/GFP. dsRNA.R}143; P{UAS-EGFP}34/+*
y^{11118}; P{ey1x-GAL4.Exel}2	Midgut salivary glands	*w; P{ey1x-GAL4.Exel}2/+; P{UAS-EGFP}34/+*	EGFP	*w; P{ey1x-GAL4.Exel}2/ P{UAS-Avic/GFP.dsRNA.R}143; P{UAS-EGFP}34/+*
w; ap^{MD544}/CyOen11	Wing discs	*w; ap^{MD544}/+; P{UAS-EGFP}34/+*	EGFP	*w; ap^{MD544}/ P{UAS-Avic/GFP.dsRNA.R} 143; P{UAS-EGFP}34/+*
w; pxn-Gal4	Hemocytes	*w; P{UAS-GFP.S65T}T2/+; pxn-Gal4/+ GFP*	GFP	N/A
w; FRT-G13, tubP-Gal80; tubPGal4/TM6B, Tb	None	*w; FRT-G13, tubP-Gal80/P {UASGFP.S65T}T2;tubP-Gal4/+.*	Gal80	N/A

The list of Gal4 lines used in this study. Genotypes of crosses and the EGFP (or GFP) expression pattern in the last larval stage are also listed. EGFP was used for most of the experiments. GFP^{S65T} was used for monitoring reporter expression in hemocytes (*pxn-Gal4*) because we could not detect a strong EGFP signal in the hemocytes.

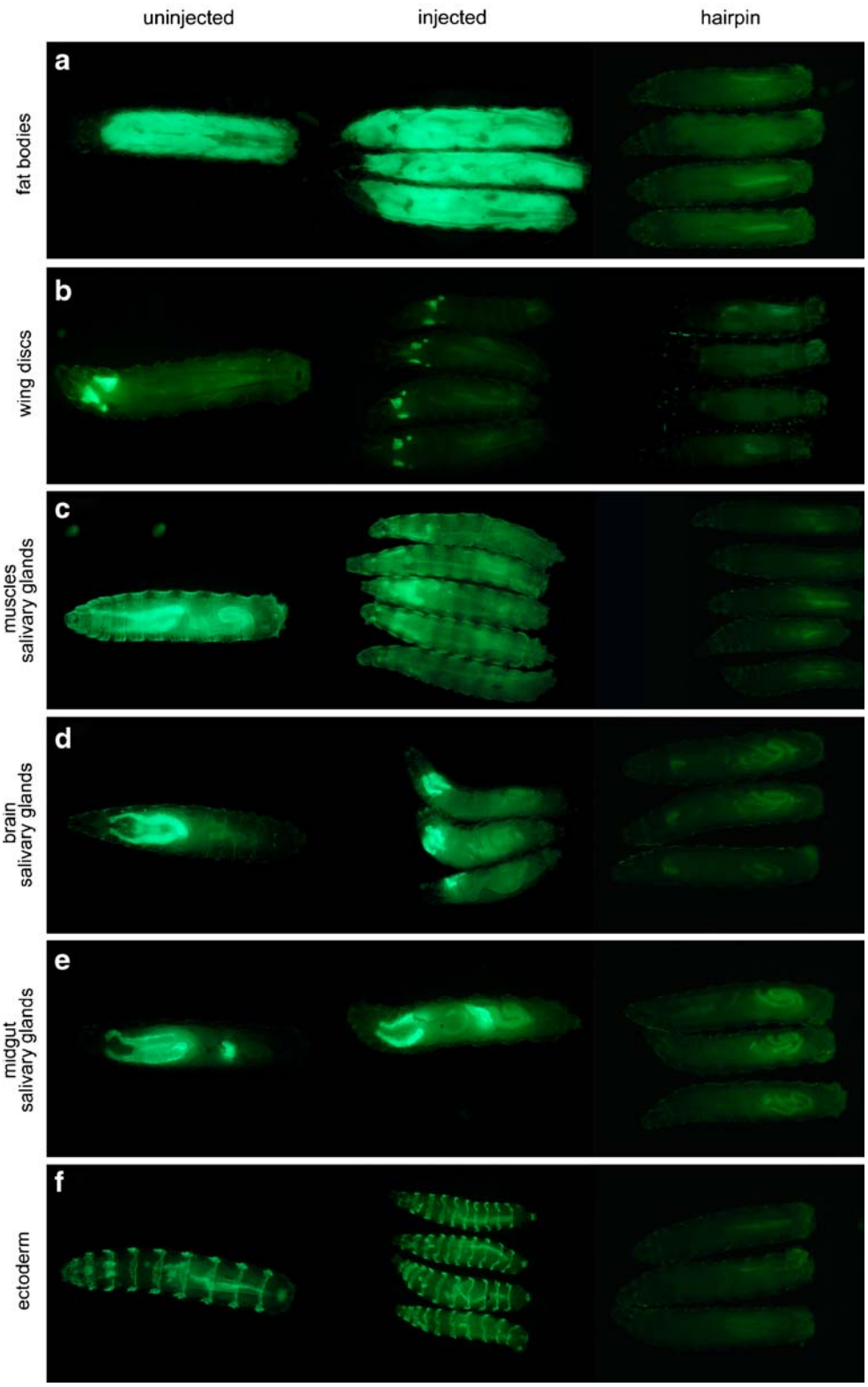

Fig. 1 Lack of a systemic RNAi response in a variety of *Drosophila* larval tissues. **a–f** *Left* EGFP expression driven by various Gal4 lines; *middle* EGFP expression 24 h after injection of EGFP dsRNA; *right* EGFP expression in EGFP hairpin RNA co-expressing flies. All *Drosophila* larvae are last larval instar, oriented anterior left, posterior right

Injection into fly larvae

Last instar larvae expressing the reporter gene were selected. Larvae were dried on filter paper and then immobilized on double-stick tape adhered to a microscope slide. The larvae were positioned ventral side down and were injected on their dorsal side in an anterior-to-posterior direction. dsRNA for EGFP and GFP was injected at a concentration of 1 μg/μl (less than 0.25 μg per larva). dsRNA for Gal80 was injected at a concentration of 2 μg/μl

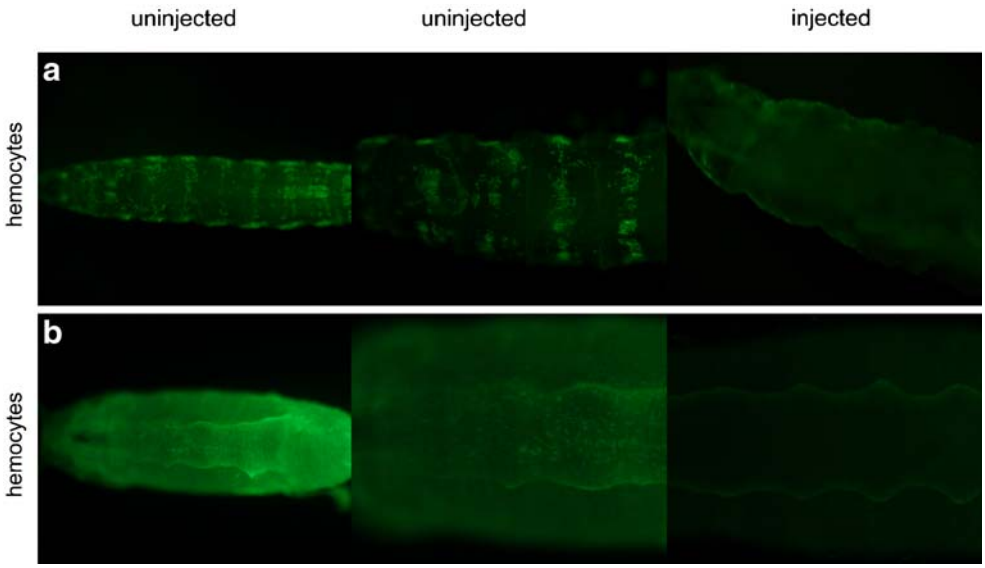

Fig. 2 Systemic RNAi response in *Drosophila* hemocytes. **a** *Left and middle* GFP expression in the hemocytes driven by *pxn-Gal4*; *right* GFP expression 24 h after injection of GFP dsRNA. **b** *Left and middle* Pupal stage GFP expression in hemocytes driven by *pxn-Gal4*; *right* pupal stage GFP expression 48 h after injection of GFP dsRNA into larvae. All *Drosophila* larvae are last larval instar. Larvae and pupae are oriented anterior left, posterior right

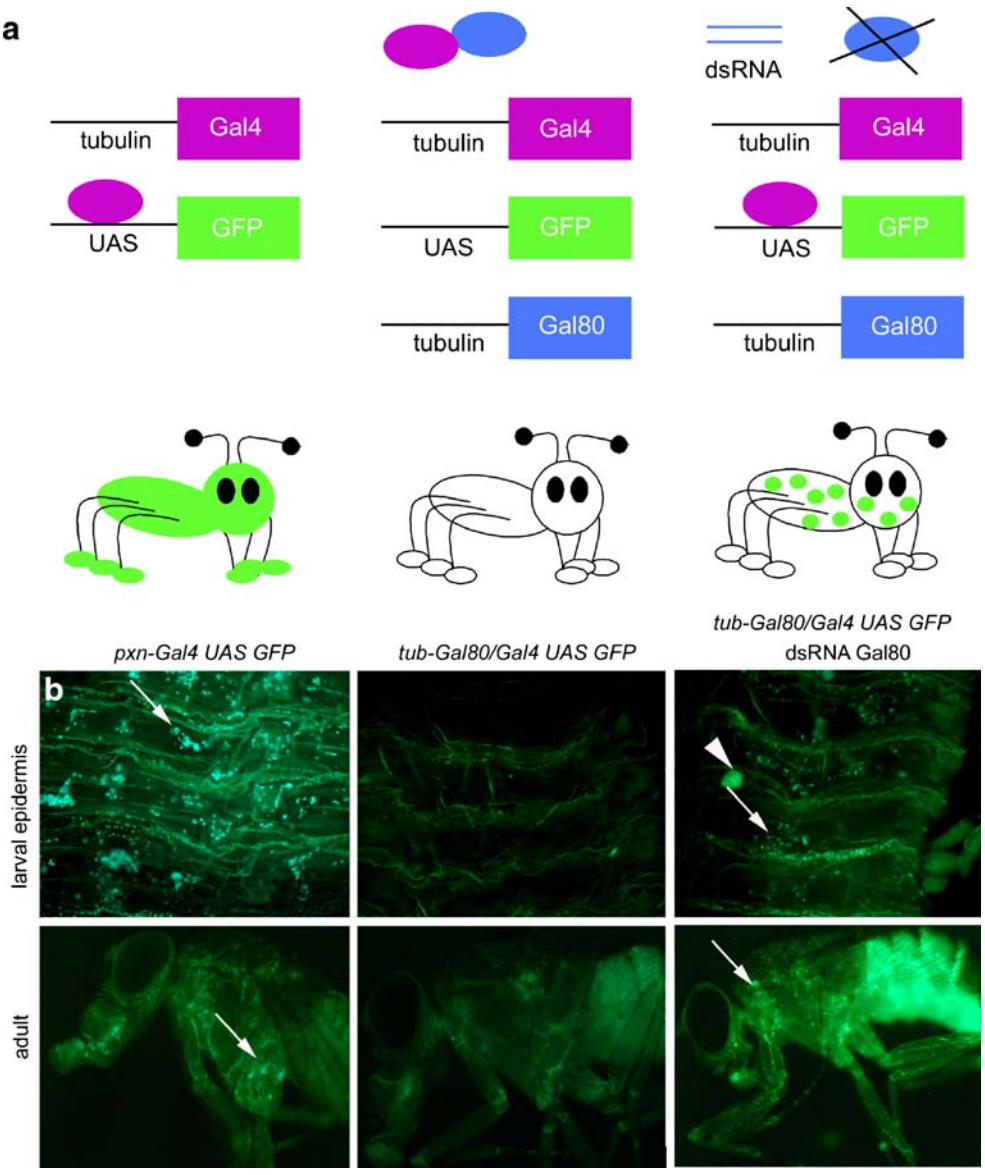

Fig. 3 Visualization of systemic RNAi-sensitive tissues in *Drosophila* larvae. **a** *Gal4/UAS* and the *Gal80/Gal4/UAS* system. *Left* The ubiquitous expression of Gal4 drives GFP expression in all tissues (*tub-Gal4 UAS-GFP*); *middle* Gal80 represses the function of Gal4 resulting in individuals with no GFP expression (*tub-Gal80/Gal4 UAS-GFP*); *right* GFP expression is only seen in tissues that take up the Gal80 dsRNA and mount an RNAi response (*tub-Gal80/Gal4 UAS-GFP* injected with Gal80 dsRNA). **b** *Left* larval epidermis and adult with GFP expression in the hemocytes driven by *pxn-Gal4*; *middle tub-Gal80/Gal4 UAS-GFP* larval cuticle and adult. No GFP expression is observed due to repression by Gal80; *right tub-Gal80/Gal4 UAS-GFP* larval epidermis and adult after larval injection of Gal80 dsRNA. *Arrows* point to GFP-expressing hemocytes. *Arrowhead* indicates injection wound

(less than 0.5 μg per larva). Larvae were removed from the slide after injection and raised at 25°C until analysis.

Results and discussion

To determine whether *Drosophila* larval cells have the ability to take up dsRNA and execute an RNAi response, we employed the Gal4/UAS system (Brand and Perrimon 1993) to express a reporter gene (GFP or EGFP) in a variety of *Drosophila* tissues (Table 1, Fig. 1a–f left, Fig. 2a–b left and middle). We then injected third-instar larvae with dsRNA for the reporter gene and monitored reporter gene expression 24 and 48 h (data not shown) after injection. Reporter gene expression was maintained in most of the tissues tested including wing discs, fat bodies, salivary glands, muscles, midgut, brain, and ectoderm (Fig. 1a–f middle). Intriguingly, reporter gene expression was lost in only one of the cell types tested, the hemocytes (Fig. 2a–b right). A mock injection (dsRNA for dsRed) did not induce the reduction of reporter gene expression in hemocytes (data not shown), indicating that the dsRNA silencing in hemocytes is not a non-specific effect induced by dsRNA molecules. Our findings indicate that introducing dsRNA by injection in the last larval stage is ineffective at triggering RNAi in many tissues. However, *Drosophila* hemocytes [visualized by *pxn-Gal4/UAS-GFP* (Stramer et al. 2005)] do appear to have the ability to take up dsRNA and perform RNAi in vivo.

To confirm our results and test other *Drosophila* tissues for a systemic RNAi response, we took advantage of the MARCM system (Lee and Luo 2001). This system uses Gal80 as a repressor of Gal4 to prevent expression normally produced by the Gal4/UAS system (Lee and Luo 2001). We created a line in which GFP expression was driven by UAS, and both Gal80 and Gal4 were expressed ubiquitously by the *tubulin* promoter (Table 1). In these flies, there is no GFP expression due to the repression of Gal4 by Gal80 (Fig. 3b middle). We attempted to relieve Gal4 repression by injecting dsRNA for Gal80 into last instar larvae. When GFP expression was monitored 24 and 72 h after injection, hemocytes were the only tissue in which Gal4 repression appeared to be relieved (Fig. 3b right). These data confirm our initial results, suggesting that hemocytes are the only apparent tissue capable of taking up dsRNA and mounting an RNAi response.

To determine whether the ineffective RNAi response in most *Drosophila* tissues was due to a lack of intracellular RNAi machinery or due to a more upstream process, we expressed EGFP hairpin RNAs to trigger RNAi inside the cells (Roignant et al. 2003; Table 1). We observed a reduction of EGFP expression in all tissues examined (Fig. 1a–f right), indicating that the lack of RNAi response after injection of dsRNA was not due to defects in the RNAi machinery. These results lend support to the conclusion that failure in upstream events in the systemic RNAi pathway, such as dsRNA cellular uptake, transport, or maintenance, is responsible for the ineffective RNAi in most *Drosophila* larval tissues.

Recent reports illustrate that post-embryonic injection of dsRNA in other non-drosophilid insects can result in a systemic RNAi effect. However, in many of these organisms, only specific tissues, such as epidermal tissues or fat bodies, have been examined (summarized in Tomoyasu et al. 2008). To reveal whether all tissues in *Tribolium* have the capacity to take up dsRNA, we utilized a transgenic *Tribolium* line in which EGFP is driven ubiquitously by the native *alpha tubulin* (*aTub*) promoter (R. Beeman, personal communication). Last-instar larvae were injected with dsRNA

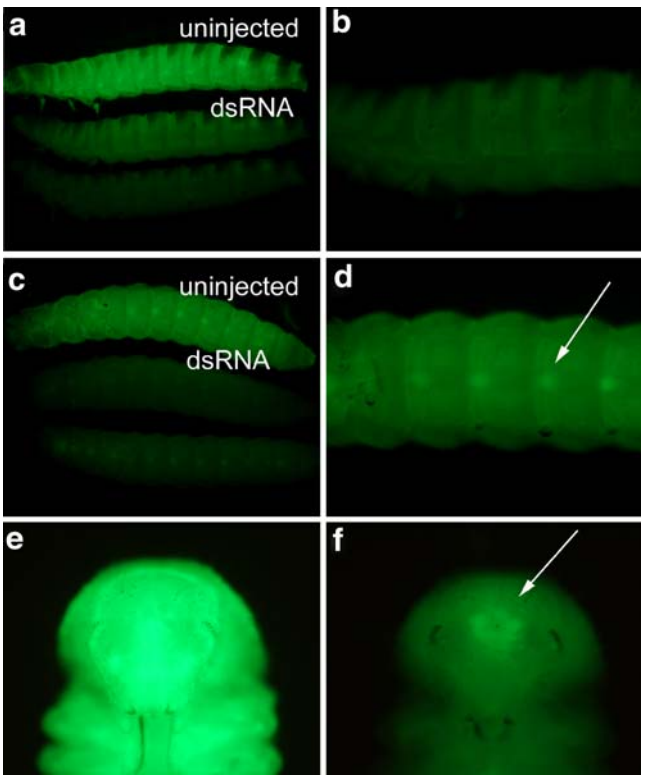

Fig. 4 Virtually all tissues are sensitive to systemic RNAi in *Tribolium* larvae. **a** Lateral view of *aTub-EGFP Tribolium* larvae. Upper larva was uninjected. Lower two larvae were injected with EGFP dsRNA. **b** Lateral view of *aTub-EGFP Tribolium* larva injected with EGFP dsRNA. **c** Ventral view of *aTub-EGFP Tribolium* larvae. Upper larva was uninjected. Lower two larvae were injected with EGFP dsRNA. **d** Ventral view of *aTub-EGFP Tribolium* larvae injected with EGFP dsRNA. **e** *aTub-EGFP Tribolium* pupa. **f** *aTub-EGFP Tribolium* pupa injected with EGFP dsRNA. *Tribolium* larvae are oriented anterior left, posterior right. EGFP expression was documented 48 h after injection of dsRNA. Pupae are shown ventral view, oriented anterior up, posterior down. EGFP expression was documented 96 h after injection of dsRNA. *Arrows* point to residual EGFP expression

for EGFP and then monitored for EGFP expression 48 and 96 h later. In contrast to the *Drosophila* results, EGFP expression was reduced or absent in virtually all *Tribolium* tissues at both larval and pupal stages (Fig. 4a–f). In most *Tribolium* tissues, EGFP expression appears completely absent. However, some residual EGFP expression is still seen in the ventral portion of each larval segment (ganglia; arrow in Fig. 4d) and in the pupal brain (arrow in Fig. 4f). The remaining EGFP expression may be due to differences in initial expression rather than tissues resistance to RNAi as both of these tissues expressed EGFP at a higher level than surrounding tissues prior to injection. The higher level of initial EGFP expression in the brain and ganglion may require more time and/or dsRNA to achieve efficient knockdown. Alternatively, there still is a possibility that some *Tribolium* neural tissues may be somewhat resistant to RNAi. These results indicate that virtually all *Tribolium* larval tissues have the ability to take up dsRNA from the extracellular environment and mount an RNAi response.

Our data provide insight into the use of RNAi as a tool for the study of post-embryonic development in insects. While larval injection of dsRNA is not effective for many *Drosophila* tissues, some successful reports of adult injection (Dzitoyeva et al. 2001; Goto et al. 2003; Petruk et al. 2006) may suggest different tissue specificity at different developmental stages. The basis of this difference between larval and adult tissues is still unknown but may be due to fundamental developmental differences between tissue types, such as cell ploidy, or due to differences in gene expression required for the uptake and transport of dsRNA. While most *Drosophila* larval tissue is not susceptible to dsRNA by injection, our data does reveal a potentially powerful application for RNAi in the study of hemocyte development and their role in insect immunity. In addition, *Tribolium*'s ability to efficiently perform RNAi in virtually all cell types makes it an attractive insect model for the study of post-embryonic development and the systemic RNAi response itself.

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