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Evidence for evolutionary constraints in *Drosophila* metal biology

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Abstract Mutations in single *Drosophila melanogaster* genes can alter total body metal accumulation. We therefore asked whether evolutionary constraints maintain biologically abundant metal ions (iron, copper, manganese and zinc) to similar concentrations in different species of Drosophilidae, or whether metal homeostasis is a highly adaptable trait as shown previously for triglyceride and glycogen storage. To avoid dietary influences, only species able to grow and reproduce on a standard laboratory medium were selected for analysis. Flame atomic absorption spectrometry was used to determine metal content in 5-days-old adult flies. Overall, the data suggest that the metallome of the nine species tested is well conserved. Meaningful average values for the Drosophilidae family are presented. Few statistically significant differences were noted for copper, manganese and zinc between species. In contrast, *Drosophila erecta* and *Drosophila virilis* showed a 50% increase above average and a 30% decrease below average in iron concentrations, respectively. The changes in total body iron content correlated with altered iron storage in intestinal ferritin stores of these species. Hence, the variability in iron content could be accounted for by a corresponding adaptation in iron storage regulation. We suggest that the relative expression of the multitude of metalloenzymes and other metal-binding proteins remains overall similar between species and likely determines relative metal abundances in the organism. The availability of a complete and annotated genome sequence of different *Drosophila* species presents opportunities to study the evolution of metal homeostasis in closely related organisms that have evolved separately for millions or dozens of million years.

Keywords Transition metals · Insect physiology · Ferritin iron stores · Metallomics

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Introduction

How genetic variability affects metal accumulation has been studied in the agricultural context of plant bio-fortification to address nutritional deficiencies in humans (Palmer and Guerinot 2009; White and Broadley 2009). A major conclusion from these studies was that soil composition had a major influence on metal incorporation into plants. Nonetheless, genetic strains of plants with significant accumulations of one or more metals in different parts of the plant have been generated. On the other hand, and although a body of work has addressed the nutritional control of metal absorption and dietary aspects of animal metal homeostasis (Lonnerdal and

Kelleher 2009; Sharp and Srai 2007; van den Berghe and Klomp 2009), less attention has been given to the identification of genetic variants with altered metal homeostasis in animals.

A century of experimental work with *Drosophila melanogaster* has largely informed our understanding of heredity and animal development. More recently, *Drosophila* research is regularly contributing the many other fields of biology. Nevertheless, generalizations of the findings with *D. melanogaster* to other animals and even other insects should require caution. Only the acalyptrate family of Drosophilidae in the insect order Diptera includes several thousand species that have evolved separately for over 40 million years (Markow and O'Grady 2007). Very limited information exists as to whether each species has adapted its general physiology differently along with other life-history traits. In one study of comparative physiology, total triglyceride, glycogen and protein were found to vary as much as tenfold between eleven species of *Drosophila* (Matzkin and Markow 2009). A lack of correlation between triglyceride and glycogen pools suggested that the two metabolic energy storage sources adapted independently during evolution (Matzkin and Markow 2009). More information on physiologic traits of the different species will undoubtedly help investigators of functional and evolutionary genomics to associate changes in DNA sequence with each organism's specific adaptations.

Recent work in metal biology, using the fruit fly, focused more particularly to the function of individual genes (Balamurugan et al. 2007; Mehta et al. 2008; Missirlis et al. 2007; Selvaraj et al. 2005; Metzendorf et al. 2009; Bettedi et al. 2011). The publication of a complete and annotated genome sequence of twelve *Drosophila* species (Clark et al. 2007) has allowed the application of comparative genomic methods to the study of genes in metal homeostasis. For example, application of *Evoprinter*, a multigenomic DNA sequence analysis tool (Odenwald et al. 2005), to the genomic locus of *Drosophila* ferritin genes allowed the identification of an iron responsive element and the binding sites of metal transcription factor-1, both evolutionarily conserved (Missirlis et al. 2007). In another example, a comparison between two highly homologous cytosolic aconitases, conserved in the different *Drosophila* genomes, enabled the identification of key amino acids that confer the ability in one of the two proteins to bind iron responsive elements (Lind et al. 2006). Mutations in single genes, such as in the *Malvolio* (*Mvl*) and *Multicopper Oxidase 3* genes, are sufficient to alter iron and copper accumulation in whole flies, respectively (Southon et al. 2008; Bettedi et al. 2011). As iron, copper, manganese and zinc are all biologically important metal ions, we wondered whether evolutionary constraints have operated to regulate their absolute and relative concentrations in different species of the Drosophilidae. To avoid dietary influences, we chose to investigate species that successfully grow and reproduce on classic laboratory medium. We used flame atomic absorption spectrometry to determine metal content in 5-days-old adult flies of each species and discuss the significance of our findings for evolutionary considerations of *Drosophila* metal homeostasis.

Materials and methods

Source of *Drosophila* species and laboratory maintenance

The stocks used in this study were obtained from the *Drosophila* Species Stock center located at the University of California, San Diego. Most of the stocks have been kept in captivity for several years, with the exception of three *Drosophila simulans* stocks originating from single female flies collected in 2009 at Lujeri, Malawi. Wild type *D. melanogaster* flies were collected in Italy by Rudi Costa in 2004 (Tauber et al. 2007). Identifying stock numbers are provided in Table 1. Flies were kept at 25°C in light–dark cycles (12 h: 12 h) on a diet containing sucrose (9.7%), glucose (21.3%), yeast (22.6%), maize meal (9.7%), treacle (19.3%), soya flour (4.6%), agar (6.5%), propionic acid (0.5%) and nipagin (0.01%).

Preparation of samples for flame atomic absorption spectrometry

As previously described (Gutierrez et al. 2010), 5-days-old adult flies of both sexes were collected, typically from 4–5 plastic vials, and killed by freezing in liquid nitrogen. Samples were freeze-dried and weighed (aiming for approximately 100 mg of material per sample). The dry samples were

Table 1 Metal content (mg metal/g dry weight) of 5-days old adult flies from the San Diego *Drosophila* Species Stock Center collection

Species	ID	Genotype	N	Fe	Cu	Mn	Zn
D. ananassae	14024-0371.00	wt	6	0.20 ± 0.03	0.012 ± 0.001	0.028 ± 0.010	0.19 ± 0.05
D. erecta	14021-0224.00	wt	7	0.33 ± 0.07	0.015 ± 0.006	0.031 ± 0.011	0.22 ± 0.10
D. erecta	14021-0224.02	y^1	4	0.29 ± 0.04	0.013 ± 0.003	0.025 ± 0.007	0.23 ± 0.07
D. melanogaster	Italian origin	wt	12	0.23 ± 0.06	0.016 ± 0.001	0.019 ± 0.005	0.16 ± 0.03
D. melanogaster	14021-0231.36	y^1*	8	0.23 ± 0.08	0.021 ± 0.003	0.022 ± 0.012	0.13 ± 0.03
D. pseudoobscura	14011-0121.00	wt	6	0.27 ± 0.02	0.021 ± 0.003	0.030 ± 0.020	0.23 ± 0.08
D. pseudoobscura	14011-0121.06	y^1	6	0.21 ± 0.04	0.021 ± 0.007	0.022 ± 0.011	0.19 ± 0.06
D. sechellia	14021-0248.01	wt	7	0.18 ± 0.07	0.023 ± 0.008	0.034 ± 0.014	0.20 ± 0.09
D. simulans	14021-0251.261	iso1**	7	0.24 ± 0.07	0.020 ± 0.006	0.034 ± 0.013	0.21 ± 0.07
D. simulans	14021-0251.261	iso2**	7	0.23 ± 0.08	0.020 ± 0.004	0.033 ± 0.015	0.19 ± 0.07
D. simulans	14021-0251.261	iso3**	6	0.21 ± 0.05	0.015 ± 0.005	0.034 ± 0.010	0.18 ± 0.06
D. virilis	15010-1051.00	wt	8	0.13 ± 0.03	0.017 ± 0.005	0.032 ± 0.010	0.13 ± 0.06
D. virilis	15010-1051.18	b^1***	7	0.17 ± 0.07	0.018 ± 0.007	0.032 ± 0.013	0.13 ± 0.09
D. virilis	15010-1051.87	y^1****	8	0.16 ± 0.05	0.021 ± 0.009	0.041 ± 0.021	0.11 ± 0.03
D. willistoni	14030-0811.24	wt	6	0.19 ± 0.04	0.020 ± 0.003	0.026 ± 0.005	0.20 ± 0.04
D. yakuba	14021-0261.00	wt	6	0.20 ± 0.05	0.013 ± 0.003	0.028 ± 0.007	0.22 ± 0.04
Species average	–	–	111	0.22 ± 0.07	0.018 ± 0.006	0.028 ± 0.013	0.18 ± 0.07

One-way ANOVA showed significant differences only in iron measurements; Tukey's test revealed these were between *D. erecta* and *D. virilis* stocks

* Full genotype y^1; $Gr22b^1$, $Gr22b^1$, cn^1, $CG3394^{R4.2}$, bw^1, sp^1; $LysC^1$, $MstProx^1$, $GstD5^1$, $Rh6^1$

** Collected wild type flies; stocks from iso-females

*** Full genotype b^1; tb^1, $gp\text{-}L2^1$; cd^1, pe^1

**** Full genotype y^1, ch^2, mt^1

dissolved and digested in 1.5 ml heated nitric acid for 2 days. All glassware (tubes and flasks) used in the elemental analysis was acid-washed to avoid contamination. Flame atomic absorption spectrometry was used to determine the levels of iron, copper, manganese and zinc. Multiple replicate samples of each species stock were measured throughout the year.

Statistical analysis of metal measurements

One-way Analysis Of Variance (ANOVA) was performed on the data presented in Table 1. Statistical differences were considered at the 95% confidence value and the Tukey's test was performed to make all pair-wise comparisons between individual stocks. This analysis showed that within the same species, there was no instance where the individual genotypes were significantly different from each other. We therefore decided to pool the data for each species and performed a one-way ANOVA and Tukey's test at the species level, the results of which are shown in Fig. 1.

Histochemical staining for iron

Prussian blue staining for histochemical detection of iron was performed as described before (Mehta et al. 2009). Intestines were dissected from wondering third-instar larvae in phosphate buffered saline (PBS) and fixed in 4% formaldehyde in PBS for 30 min. Following washes with PBS, the tissue was permeabilized by treating with 1% Tween in PBS for 15 min. For detection of ferric iron, the samples were incubated in the dark with Prussian blue stain [2% $K_4Fe(CN)_6$, 2% HCl] for 45 min. Five washes with water followed and the preparations were mounted in water on glass slides and imaged directly on a Leitz Orthoplan microscope coupled to an Olympus DP71 camera.

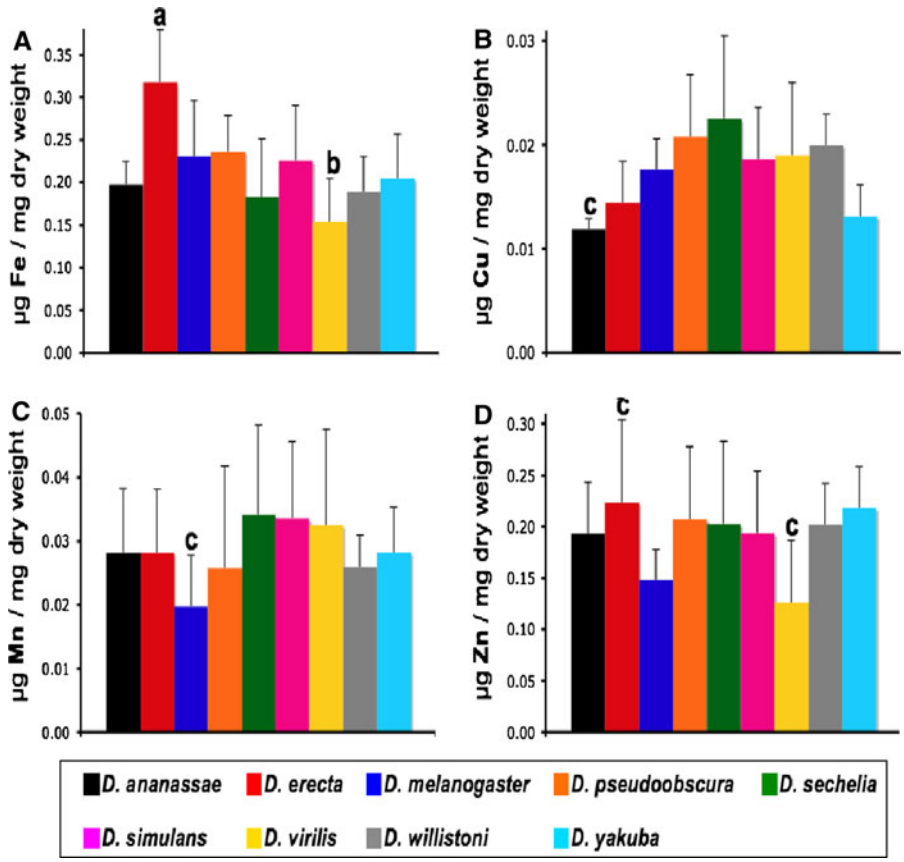

Fig. 1 Total body metal accumulations in different *Drosophila* species. Each species is represented by a different *colour bar* as indicated in the *bottom part* of the figure and each panel shows the average value and standard deviations for the four different metals measured in this study. One-way ANOVA followed by Tukey's test for pair-wise significant differences was performed. **A** Iron content per body mass is statistically significantly higher in *D. erecta* compared to all other species and significantly lower in *D. virilis* compared to *D. erecta, D. melanogaster, D. pseudoobscura* and *D. simulans*. **B** *D. ananassae* was low on copper compared to *D. pseudoobscura* and *D. sechelia*. **C** *D. melanogaster* was low on manganese compared to *D. simulans* and *D. virilis*. **D** *D. erecta* was high on zinc compared to *D. virilis* and *D. melanogaster*; the former was found low in zinc also in comparison with *D. pseudoobscura*. *a* significantly different ($P < 0.05$) than all other species; *b* significantly different than four other species; *c* significantly different than two other species

Male fertility assay

Drosophila virilis flies were raised on normal diet or an iron-enriched diet by addition of 1 mM ferric ammonium citrate (FAC). 6-days old female virgins (at this age they should be fully sexually mature; Pitnick et al. 1995; Markow and O'Grady 2007), grown on normal diet, were collected for testing male fertility. Male flies from all three diets were tested in single crosses at different times during their lifespan. Male flies were successively allowed for 24 h in vials with two females at the age of 4, 6, 9, 11 and 15 days old. Females were kept in vials for a few days to lay eggs and the appearance of viable progeny was monitored. Adult crossings were all performed under normal dietary conditions; this decision was based on our observations with *D. melanogaster* that dietary conditions during larval stages largely determine iron concentrations in adult flies (FM, unpublished data).

Results and discussion

In this study, we selected sixteen stocks of *Drosophila* flies, representing nine different species for which the genomes are available, and measured the amount of elemental iron, copper, manganese and zinc present in 5-days-old adult flies of mixed sex (Table 1).

We selected to compare species for which there is available genomic information and which are able to grow on a standard cornmeal diet, thereby excluding differences in metal concentrations due to dietary factors. Wild type flies were used for all species. In addition, we decided to include four stocks of different *Drosophila* species each one of which carried mutations in the *yellow* gene. These strains served to validate metal measurements from wild type flies. Our experimental design also included a control for any unanticipated effect of the *yellow* gene on metal homeostasis, namely a *D. virilis* stock with multiple mutations in other genes (Table 1). We also controlled for variability of metals arising from sampling within a wild population, using three recently established stocks of *D. simulans* iso-females collected at the same location. Analysis of the results in Table 1 showed in no instance statistically significant variation between different stocks of the same species. Therefore, we felt justified to pursue an analysis at the species level, by treating all the measurements in a species represented in the study by more than one strain as one group (Fig. 1).

Statistical analysis was performed to ask if there were significant differences between species in the mean values determined for each metal. One-way ANOVA suggested statistically significant differences existed ($P < 0.001$ for iron and for zinc, $P < 0.002$ for copper and $P < 0.04$ for manganese). Tukey's test was then used to identify significant differences in all pair-wise comparisons. There was only one instance in which a given species was shown to have significantly different metal content from all other species: *D. erecta* had a significantly higher content of iron (0.32 ± 0.06 μg Fe per mg dry weight; Fig. 1a), representing a 50% increase compared to the average value calculated for the family (0.22 ± 0.07; Table 1). Conversely, *D. virilis* had the lowest content of iron (0.15 ± 0.05 μg Fe per mg dry weight), a 30% decrease compared to the average value calculated for the family. The latter result was statistically significant when *D. virilis* was compared to four other species (named in the legend of Fig. 1a). We further investigated flies from both species for adaptations that might explain this difference in iron concentrations and also for particular sensitivities to dietary iron treatments (see below).

Tukey's test on the data collected from measurements of copper in the different species suggested that *D. ananassae* accumulated lower amounts of copper compared to *D. pseudoobscura* and *D. sechelia* (Fig. 1b; Table 1). This result was particularly intriguing, because *D. ananassae* was previously shown to differ from other *Drosophila* species in characteristically generating enhanced orange fluorescence under UV light, when fed on increasing doses of copper (Poulson and Bowen 1952). This fluorescence results from a copper-metallothionein complex (McNulty et al. 2001). Hence, it appears that *D. ananassae*, the very species found more prone to activate a defense response against dietary copper, normally accumulates a lower content of copper under laboratory conditions.

When looking at the data of manganese measurements, *D. melanogaster* with lower concentrations reached in average appeared to be the exception in the family (Fig. 1c). This result, which only reached statistical significance when *D. melanogaster* was compared to *D. simulans* and *D. virilis*, should however be kept in mind, since *D. melanogaster* is the key organism studied from the family by the research community and therefore processes affected by manganese may be altered in this species. Finally, when zinc concentrations were considered, *D. virilis* had low zinc concentration and *D. erecta* high, in a pattern that was reminiscent of iron (Fig. 1d). We therefore took a closer look at these two species.

We tested whether iron supplementation (1 mM FAC) affected the development of *D. erecta* and *D. virilis*, but did not find it had any impact on the timing of development or the survival of flies (data not shown). Iron (Metzendorf and Lind 2010) and copper (Steiger et al. 2010) were recently reported to be crucial for fertility. Curiously, *D. virilis* males take 6–9 days to mature sexually instead of 1–2 days as the other species used in this study (Pitnick et al. 1995; Markow and O'Grady 2007). Therefore, we also tested whether iron supplementation had a positive effect on male maturation and fertility in this species. We compared *D. virilis* males derived from larvae hatching on a diet supplemented with 1 mM FAC to males raised on normal diet. We crossed each male, at 4, 6, 9, 11 and 15 days after emergence from pupa, always to a couple of 6-days old, sexually mature, virgin females and scored the crosses for progeny (Table 2). In our hands, 4-days old males were not sexually mature (i.e. no progeny developed from these crosses) and only 40% of

Table 2 Male fertility in *Drosophila virilis* fed on diets with varying iron bioavailability

Adult age (days)	Experiments	Individuals tested	Male fertility Normal diet (%)	Male fertility 1 mM FAC (%)
4	1	22	0	0
6	3	82	41	37
9	3	82	44	56
11	2	51	37	80
15	2	59	64	83

6-days old *D. virilis* males were fertile regardless of diet. Interestingly, iron supplementation appeared to enhance the percentage of fertile males as flies aged (Table 2). We looked for, but did not find, evidence whether iron affects the speed of testis maturation. Neither did the dissected testes show obvious morphological differences between the two groups. Nevertheless, our results suggest that the relative iron deficiency associated with *D. virilis* males (compared to other Drosophilidae) may affect fertility of the offspring. This finding has significant evolutionary implications for the species if confirmed in the wild. It would therefore be interesting to test the natural feeding environments of *D. virilis* and *D. erecta* for iron bioavailability and to ask whether differences in metal accumulation persist in natural populations of *D. virilis* and *D. erecta*. For *D. virilis*, it would also be interesting to identify populations in the field feeding on diets with different iron content to test if such differences would impact on male fertility, i.e. whether our findings in the laboratory have any bearing in the natural habitats and evolution of this species. We hope to pursue these questions in future work.

Overall, our metal measurements suggest a remarkable extent of conservation in metal content in species that have evolved separately for many million years, further suggesting tight evolutionary constraints on operational and regulatory systems, which ensure that each metal is present within a specific threshold. We speculate that the specific value for each metal reflects the amount of utilization of metals as co-factors in proteins. This hypothesis fits well with the observation that copper and manganese, which are tightly bound to only a handful of proteins, are present at a level that is a magnitude lower level compared to iron and zinc, each of which is essential for the function of hundreds of proteins (Palmer and Guerinot 2009).

How then would one account for *D. erecta* and *D. virilis* accumulating different iron concentrations without affecting the function of related metalloproteins? We postulated that variations of metal storage, instead of alterations in metalloproteins involved in metabolism, might be a simpler adaptation to a given ecological environment. We tested this hypothesis for the variations in iron accumulation noted in *D. virilis* and *D. erecta*. To this end we dissected the larval intestine from all species and stained it for iron. In *D. melanogaster*, two regions of the larval intestine are known to accumulate iron: the iron region of the middle midgut, which functions in systemic ferritin iron storage, and the anterior midgut, which accumulates iron-loaded ferritin when iron is high in the diet (Missirlis et al. 2007; Mehta et al. 2009). When flies were grown on iron-supplemented media, both regions were identified by Prussian blue staining in all nine species included in this study. In contrast, when grown on the normal diet, two of the nine species showed variations of ferritin iron staining. *Drosophila virilis* had markedly lower iron stored in the iron region, and also fewer anterior midgut cells stained for iron (Fig. 2a, d). The iron deficiency associated with *D. virilis* has features reminiscent of the iron-deficient *D. melanogaster Mvl* mutants, i.e. low iron accumulation in the middle midgut but retaining the ability to accumulate iron-loaded ferritin in the anterior midgut (Bettedi et al. 2011). In contrast, *D. erecta* showed enhanced staining for iron in the iron region and a full induction of iron-loaded ferritin in the anterior midgut, as flies on a high iron diet do (Fig. 2c, f). We would predict that *D. erecta* has adapted to a nutritional environment of low iron bioavailability, as it activates ferritin expression and accumulates high iron levels at a significantly lower threshold of dietary iron than other *Drosophila* species. All other species were indistinguishable from *D. melanogaster*, showing intermediate levels of stain only in the iron region (Fig. 2b) but not in the anterior midgut (Fig. 2e). Collectively, these results corroborated our findings of total body iron measurements and offered an explanation for the difference in iron levels measured in these species, namely that they reflected changes in iron storage levels.

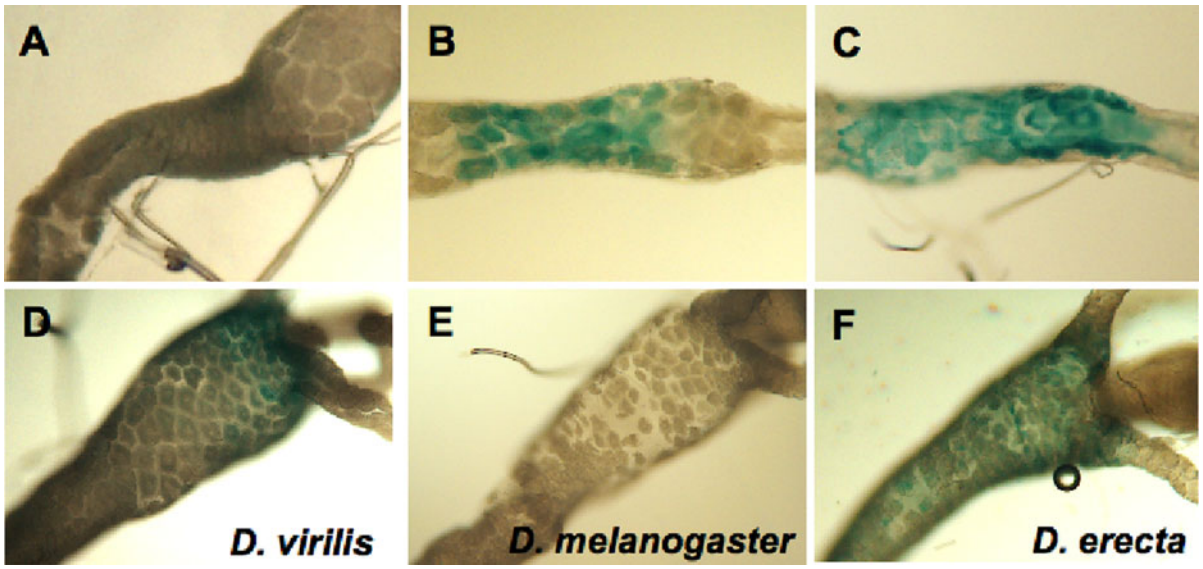

Fig. 2 The iron region of the middle midgut is conserved in different species of Drosophilidae but the amount of iron stored can vary amongst them under similar dietary conditions. Intestines were dissected from third instar wandering larvae grown on a normal diet and stained with *Prussian blue* to detect ferritin iron. **a** *Drosophila virilis* has considerably less iron in its tissue-stores compared to **b** *Drosophila melanogaster*, which represented typical levels found in most species, and **c** *Drosophila erecta*, which appeared to accumulate more iron in the iron region. **d** Iron accumulated in few cells of the anterior midgut in *Drosophila virilis*, **e** but not in *Drosophila melanogaster*. **f** The anterior midgut of *Drosophila erecta* was iron-loaded

Conclusion

We show that metal homeostasis in nine species of *Drosophila* is well conserved, at least when reared in the laboratory under similar conditions. The results suggest that the combination of the multitude of metalloenzymes and other metal-binding proteins remains well regulated between species, if our suggestion that total metal accumulation reflects the need for protein metallation is accepted. Variability of metal accumulation can be achieved via changes of metal storage control, though apparently such changes have been modest in the species tested. The extent to which relative levels of metals are affected in wild populations and in different orders of insects will require further work to confirm the conclusions of this investigation. Such work benefits from the availability of detailed genomic information.

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